Mechanistic Studies of Two Selected Flavin-Dependent Enzymes: Choline Oxidase and D-Arginine Dehydrogenase

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MECHANISTIC STUDIES OF TWO SELECTED FLAVIN-DEPENDENT ENZYMES:
CHOLINE OXIDASE AND D-ARGININE DEHYDROGENASE

by

HONGLING YUAN

Under the Direction of Giovanni Gadda

ABSTRACT

Choline oxidase catalyzes the flavin-dependent, two-step oxidation of choline to glycine betaine via the formation of an aldehyde intermediate. The oxidation of choline includes two reductive half-reactions followed by oxidative half-reactions. In the first oxidation reaction, the alcohol substrate is activated to its alkoxide via proton abstraction and oxidized via transfer of a hydride from the alkoxide \(\alpha\)-carbon to the N(5) atom of the enzyme-bound flavin. In the wild-type enzyme, proton and hydride transfers are mechanistically and kinetically uncoupled.

The role of Ser101 was investigated in this dissertation. Replacement of Ser101 with threonine, alanine, cysteine, or valine demonstrated the importance of the hydroxyl group of Ser101 in proton abstraction and in hydride transfer. Moreover, the kinetic studies on the Ser101Ala variant have revealed the importance of a specific residue for the optimization of the overall turnover of choline oxidase. The UV-visible absorbance of Ser101Cys suggests Cys101 can form an adduct with the C4a atom of the flavin. The mechanism of formation of the C4a-cysteiny1 adduct has been elucidated.
D-arginine dehydrogenase (DADH) catalyzes the oxidation of D-amino acids to the corresponding imino acids, which are non-enzymatically hydrolyzed to α-keto acids and ammonia. The enzyme is strict dehydrogenase and does not react with molecular oxygen. Steady state kinetic studies with D-arginine and D-histidine as a substrate and PMS as the electron acceptor has been investigated. The enzyme has broad substrate specificity for D-amino acids except aspartate, glutamate and glycine, with preference for arginine and lysine. Leucine is the slowest substrate in which steady state kinetic parameters can be obtained. The chemical mechanism of leucine dehydrogenation catalyzed by DADH was explored with a combination of pH, substrate and solvent kinetic isotope effects (KIE) and proton inventories by using rapid kinetics in a stopped-flow spectrophotometer. The data are discussed in the context of the crystallographic structures at high resolutions (<1.3 Å) of the enzyme in complex with iminoarginine or iminohistidine.

INDEX WORDS: Choline oxidase, Hydroxyl group, C4a-cysteinyl adduct, D-arginine dehydrogenase, Conformational change, Hydride transfer
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CHOLINE OXIDASE AND D-ARGININE DEHYDROGENASE

by

HONGLING YUAN

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CHOLINE OXIDASE AND D-ARGININE DEHYDROGENASE

by

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CHAPTER 1

Introduction

1.1 Flavoproteins

Flavoproteins contain either flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) or both as a cofactor. The core function group of flavins is the isoalloxazine ring, as shown in Figure 1.1. Flavoproteins are important in metabolic pathways, such as DNA repair, halogenations, light emission, protein folding and chromatin remodeling, as shown in Scheme 1.1 (1). Flavoproteins can transfer one or two electrons in the reaction. Therefore, a flavin can exist in three redox states: oxidized, semiquinone (one-electron reduced) and hydroquinone (two-electron reduced) (2), as shown in Figure 1.2. Each different state shows spectroscopic properties which allow kinetic studies of flavoproteins. The UV-visible absorbance spectrum of the oxidized flavin shows bands at ~ 360 nm and ~ 450 nm. For the neutral semiquinone, a broad absorbance at long wavelength, 580-620 nm, has been observed whereas the anionic semiquinone shows a strong absorbance around 380 nm and an extra sharp peak at 490 nm (3, 4). The neutral hydroquinone has characteristic absorbance at ~ 295 nm and ~ 395 nm whereas the anionic hydroquinone has absorbance around 285 nm and 340 nm (5).

![Figure 1.1](image)

Figure 1.1 The structures of isoalloxazine, FMN and FAD. Modified from ref. (6).
Flavoenzymes catalyze a variety of reactions in which the enzyme is reduced by the substrate and the turnover is completed with the oxidation of flavin by an electron acceptor, as shown in Scheme 1.2. Based on the electron acceptor, the enzymes with molecular oxygen as electron acceptor and hydrogen peroxide as product are named oxidase. Those using other electron acceptors instead of oxygen are dehydrogenases or transferases. Monooxygenases insert a single oxygen atom into the substrate and form a transient C4a-hydroperoxide intermediate.
1.2 Mechanisms of Oxidation of Carbon-Heteroatom Bond

Many flavoenzymes catalyze the oxidation of a carbon-heteroatom bond, including oxidation of alcohols, α-hydroxy acids, α-amino acids and amines (Scheme 1.3). The oxidation of alcohols has been shown to be consistent with a hydride transfer mechanism. The hydroxyl proton of the substrate is abstracted by an active site base and a hydride ion is transferred from the substrate α-carbon to the flavin N5 atom (8, 9), as shown in Scheme 1.4a. The reaction can be catalyzed by the GMC oxidoreductase enzyme superfamily, which includes choline oxidase (10, 11), pyranose 2-oxidase (12), glucose oxidase (13, 14), cholestrerol oxidase (15), methanol oxidase (16) and cellobiose dehydrogenase (17).

Scheme 1.3 Oxidation of Carbon-Heteroatom Bonds Catalyzed by Flavoenzymes.
Scheme 1.4 Proposed Mechanisms of Flavin Reduction Catalyzed by Flavoenzymes.
In contrast, the chemical mechanism of amine oxidation by flavoenzymes, such as D-amino acid oxidase (DAAO) and monoamine oxidase (MAO), has been debated for years. In addition to the hydride transfer mechanism, several mechanisms were also proposed for flavoenzyme reductive half-reactions. As shown in Scheme 1.4b, the carbanion mechanism involves the formation of a carbanionic intermediate after abstraction of the substrate α-proton (18, 19). In the radical mechanism, a single-electron is transferred from the amine nitrogen to the flavin and the substrate α-proton is removed forming a carbanion radical that transfers a second electron to the flavin (Scheme 1.4c) (20, 21). It has been proposed that in the nucleophilic mechanism amine attacks the C4a position of the flavin, leading to the formation of a C4a adduct intermediate (Scheme 1.4d) (22).

The carbanion mechanism is proposed based on the reaction of oxidation of β-Cl-alanine by DAAO (18, 19). As shown in Scheme 1.5, the observation of the elimination of HCl and the oxidation of β-Cl-alanine to β-Cl-pyruvate suggests the formation of a carbanion intermediate. Moreover, the same substrate isotope effects with α-3H-Cl-alanine on the formation of β-Cl-pyruvate and pyruvate support the conclusion that a C-H bond cleavage occurs in a common intermediate.

![Scheme 1.5 Proposed Carbanion Mechanism of β-Cl-Alanine with DAAO.](image)

However, substrate isotope effect studies of DAAO containing 5-deazaFAD with α-3H-alanine showed that the reduced flavin at N5 position was tritium-labeled, which suggested
hydride from the substrate α-carbon was transferred to the modified form of the cofactor (23, 24). The crystal structures also support a hydride transfer mechanism when pig kidney DAAO structures were solved in 1996 (25, 26). Based on crystal structure, there is no active site base which is required in carbanion mechanism properly placed to abstract the substrate α-carbon. The isotope effects studies on DAAO carried out by Fitzpatrick’s group also show that the reaction mechanism is consistent with hydride transfer (27-29). First, the pH dependence of the $^{15}$N isotope effect on the $k_{cat}/K_m$ values with D-serine as a substrate suggested that a proton was released from free D-serine to the enzyme-bound amino acid and it is at least a partially rate-limiting step (27-29). However, lack of solvent isotope effect on the $k_{cat}/K_m$ indicated that the proton released from D-serine does not occur when the C-H bond is cleaved (28). Therefore, they proposed that there is a proton released in a pre-equilibrium state and a hydride transfer from the α-carbon atom of the anionic amine to the flavin N(5) atom as shown in Scheme 1.6. Computational studies on the free energy profile of the reaction and the electronic distribution have also supported a hydride transfer mechanism from the anionic amino acid to the flavin rather than a carbanion mechanism (30).

![Scheme 1.6 Proposed Hydride Transfer Mechanism of DAAO.](image)

Two other mechanisms for the reductive half reaction have been proposed based on the studies of MAO: a radical mechanism and a nucleophilic mechanism. The radical mechanism is supported by the inhibition studies of MAO with cyclopropyl or cyclobutyl rings from McEwen
(31), Paech (32) and Silverman (20, 21) groups. The studies show that the inhibitor bound either to the protein or the flavin to inactive the MAO. Silverman (20, 21) concluded that the inactivation mechanism occurred by one electron transfer from the inhibitor to the flavin N5 atom, which indicated the formation of radical intermediate, as shown in Scheme 1.4c. However, the studies with trans-2-phenyl(aminomethyl)-cyclopropane showed the radical intermediate is not required for the inactivation of MAO (33, 34). Moreover, the lack of flavin semiquinone UV-visible spectrum in the stopped-flow experiment (35, 36) and radical spectrum in EPR (37) suggested that radical mechanism is less tenable. Also, the radical intermediate mechanism became even weaker when the flavin N5 adduct was observed in the crystal structure of MAO with cyclopropyl ring (38).

An alternative mechanism, nucleophilic mechanism, was proposed by Hamilton based on model chemistry (22). According to the crystal structure of MAO with cyclopropyl ring, this mechanism was supported by Edmondson (38, 39). However, there are also several observations that are not consistent with the nucleophilic mechanism. First, the large kinetic isotope effect with deuterium benzylamine and the absence of an C4a adduct intermediate (40) implied the formation of the adduct is reversible and sufficiently unfavorable. Second, the rate constants for reduction of MAO A in a series of ring-substituted benzylamine are sensitive to the volume instead of the electron donating ability of the benzylamine (41). Therefore, based on these studies, the mechanism for primary amine oxidation catalyzed by flavin-dependent enzymes is still controversial.
1.3 Protein Hydroxyl Group Proximal to the C(4a)-N(5) Flavin Atoms

1.3.1 Physical Properties of Hydroxyl Group

Amino acids with a hydroxyl group, including serine, threonine and tyrosine, are polar, uncharged at physiological pH, and hydrophilic. The hydroxyl group enables them to form hydrogen bonds in a protein structure. As shown in Scheme 1.7, serine and threonine have hydroxyl groups attached to aliphatic side chains, which make them more hydrophilic. The hydroxyl groups on sp$^3$-hybridized carbons can be a donor and two acceptors in the formation of hydrogen bond. Compared with serine, threonine is less polar, sterically more hindered and chemically less reactive since it has one more methyl group than serine. Tyrosine has a hydroxyl group attached to the benzene ring. The hydroxyl group on sp$^2$-hybridized carbon can only be a donor and an acceptor in the formation of hydrogen bond since the R-OH bond has partial double-bonded character (42). The phenolic hydroxyl group makes tyrosine reactive and imparts a significant degree of polarity. The pK$_a$ of the hydroxyl groups of serine and threonine are very high with values about 15 and not easy to ionize. The phenolic hydroxyl of tyrosine with a pK$_a$ of $\sim$10 is significantly more acidic than the aliphatic hydroxyls of either serine or threonine.

![Scheme 1.7 The Structure of Hydroxyl Amino Acids.](image-url)
1.3.2 Conserved Hydroxyl Group in Flavoenzymes

According to the crystal structure of flavoenzymes that are available in the PDB (protein data bank) and kinetic studies, amino acid residues with hydroxyl groups are highly conserved in proximity to the flavin C(4a)-N(5) atoms and play an important role in the flavin reduction and oxidation reactions (Table 1.1).

Table 1.1 List of Flavoenzymes with Hydroxyl Group in the Active Site.

<table>
<thead>
<tr>
<th>enzymes</th>
<th>residues</th>
<th>PDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>choline oxidase</td>
<td>Ser101</td>
<td>2jbv</td>
</tr>
<tr>
<td>D-arginine dehydrogenase</td>
<td>Tyr249/53</td>
<td>3nye</td>
</tr>
<tr>
<td>Pyranose 2-oxidase</td>
<td>Thr169</td>
<td>1tt0</td>
</tr>
<tr>
<td>NADPH-cytochrome P450 oxidoreductase</td>
<td>Ser457</td>
<td>1amo</td>
</tr>
<tr>
<td>ferredoxin-NADP⁺ reductase</td>
<td>Ser96</td>
<td>1frn</td>
</tr>
<tr>
<td>old yellow enzyme</td>
<td>Thr37</td>
<td>1oyb</td>
</tr>
<tr>
<td>alditol oxidase</td>
<td>Ser106</td>
<td>2vfs</td>
</tr>
<tr>
<td>glucose oxidase</td>
<td>Thr110</td>
<td>1cf3</td>
</tr>
<tr>
<td>pentaerythritol tetratranitate reductase</td>
<td>Thr26</td>
<td>1h51</td>
</tr>
<tr>
<td>morphphinone reductase</td>
<td>Thr32</td>
<td>1gwj</td>
</tr>
<tr>
<td>S-mandelate dehydrogenase</td>
<td>Ser108/Tyr131</td>
<td>1huv</td>
</tr>
<tr>
<td>human glycolate oxidase</td>
<td>Tyr132/26</td>
<td>2rdu</td>
</tr>
<tr>
<td>flavocytochrome b2</td>
<td>Tyr254/143</td>
<td>1fcb</td>
</tr>
<tr>
<td>porcine D-amino acid oxidase</td>
<td>Tyr228/224/Thr317</td>
<td>1kif</td>
</tr>
<tr>
<td>L-lactate oxidase</td>
<td>Tyr146/40</td>
<td>2du2</td>
</tr>
<tr>
<td>chorismate synthase</td>
<td>Ser127/16</td>
<td>1qxo</td>
</tr>
<tr>
<td>yeast D-amino acid oxidase</td>
<td>Ser335/Tyr338</td>
<td>1cop</td>
</tr>
<tr>
<td>class 2 dihydroorotate dehydrogenase</td>
<td>Ser175/Thr178</td>
<td>1f78</td>
</tr>
<tr>
<td>monomeric sacrosine oxidase</td>
<td>Thr48/Tyr317</td>
<td>1el5</td>
</tr>
<tr>
<td>heterotetrameric sacrosine oxidase</td>
<td>Thr66</td>
<td>2gag</td>
</tr>
<tr>
<td>aryl-alcohol oxidase</td>
<td>Tyr92</td>
<td>3fim</td>
</tr>
<tr>
<td>nitroalkane oxidase</td>
<td>Ser267/ser171</td>
<td>3d9g</td>
</tr>
<tr>
<td>aklavinone-11-hydroxylase</td>
<td>Tyr224</td>
<td>3ihg</td>
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<tr>
<td>2-nitropropane dioxygenase</td>
<td>Ser288</td>
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</tr>
<tr>
<td>p-hydroxybenzoate hydroxylase</td>
<td>Ser212/Tyr201</td>
<td>1dod</td>
</tr>
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<td>glycine oxidase</td>
<td>Tyr246</td>
<td>1ry1</td>
</tr>
<tr>
<td>chromate reductase</td>
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</tr>
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<td>Tyr429</td>
<td>1zr6</td>
</tr>
<tr>
<td>medium chain acyl-CoA dehydrogenase</td>
<td>Thr136/168</td>
<td>1udy</td>
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<td>porcine cytochrome b5 reductase</td>
<td>Ser99/tyr65</td>
<td>1ndh</td>
</tr>
<tr>
<td>trimethylamine dehydrogenase</td>
<td>Tyr169</td>
<td>1djn</td>
</tr>
<tr>
<td>human monoamine oxidase B</td>
<td>Tyr435</td>
<td>1gos</td>
</tr>
</tbody>
</table>
1.3.2.1 Stabilization of the Transition State or Reaction Intermediate

The transition state is the most unstable state in the reaction pathway as the chemical bonds are in the process of being made and broken. Transition state theory for enzyme-catalyzed reactions proposes that catalysis needs tight-binding at the transition state (43, 44). The binding forces of the transition state can be affected by hydrogen and ionic bond energy on bond distance, angle and solvent environment. Dynamic electronic environments and hydrogen bonding network in enzymatic catalytic site increase the probability of forming the transition state (45). Changes made at the active site could fail to stabilize the transition state. In the flavoenzymes, the hydroxyl group in the active site can form a hydrogen bond with flavin or substrate in the transition state. Removal of the hydroxyl group could destroy the stabilization of transition state.

In pyranose-2 oxidase, Thr169 sits above the flavin si-side, as shown in Figure 1.3. Steady state and transient kinetics of wild-type, Thr169Ser, Thr169Asp, Thr169Gly and Thr169Ala variants with D-glucose or D-galactose as a substrate suggest that Thr169 is important to stabilize the C4a-hydroperoxy-FAD intermediate through its interaction with the flavin N5/O4 locus (46). The oxidative half-reaction of wild-type carried out in stopped-flow instrument shows that a transient intermediate is observed (47). However, there is no intermediate detected in any of the Thr169 variants (46). In addition, the crystal structure of wild-type, Thr169Ser and Thr169Gly also showed that the side chains of Thr169 form an H-bond with the N5/O4 locus.
Ser457 in NADPH-cytochrome P450 oxidoreductase was also studied with isotope effect on the reductive half-reaction of the alanine, cysteine and threonine variants (48). The 100-fold decrease of the flavin reduction rate constant and more than 2-fold increase of the primary substrate KIE in Ser457Ala suggests an increase in the rate limiting of hydride transfer. The steady state kinetics and flavin content studies show that the mutation has no effect on cofactor binding. Furthermore, the decrease of the redox potential of FAD semiquinone in the Ser457Ala mutant suggests the inability to stabilize the FAD semiquinone. The results suggest that the Ser457 is important to stabilize the transition state for hydride transfer by H-bonding interaction.

Ser96Val variant of ferredoxin-NADP⁺ reductase folds properly based on the UV-visible, fluorescense, crystal structure and circular dichroism spectra. However, the turnover number and second-order rate constant ($k_{cat}/K_m$) are significantly different from wild-type enzyme. Also, there is no charge-transfer species accumulated in rapid reaction of Ser96Val variant as suggested by little spectral change at 700 nm during the reduction of the enzyme by NADPH. Thus, the removal of hydroxyl group results the loss of interaction between nicotinamide and flavin which disrupt the transition state during the hydride transfer between nicotinamide and

**Figure 1.3** The active site of pyranose-2 oxidase (PDB code 1TT0).
FAD. The absence of neutral semiquinone in Ser96Val variant in the photoreduction also shows the destabilization of the semiquinone (49).

In the old yellow enzyme family, Thr37 is conserved and proposed to be H-bonded with C(4)=O based on the crystal structure (50, 51). However, the only evidence is the decreased redox potential in the Thr37Ala variant compared to wild-type. According to the crystal structure and sequence alignment, the hydroxyl protein residue is conserved in several flavoenzymes which are proposed to form a H-bond with either N5 or C(4)=O of flavin, such as Ser106 in alditol oxidase (52), Thr110 in glucose oxidase (14), Thr26 in pentaerythritol tetranitrate reductase (53), Thr32 in morphinone reductase (53), Thr24 in xenobiotic reductase B (53), Thr24 in glycerol trinitrate reductase (53), Thr48 in estrogen-binding proton (53), Thr32 in 12-oxophytidienoic acid reductase (53), Ser105 in long chain hydroxy acid oxidase (54), Ser108 in S-mandelate dehydrogenase (55), Ser106 in spinach glycolate oxidase (55).

Also, the hydroxyl protein group can stabilize the transition state by H-bonding with the substrate, as shown in Figure 1.4. Several enzymes and the corresponding variants have been studied. The Lederer’s group studied the Tyr254Phe and Tyr254Leu mutants of flavocytochrome b2 (56, 57). The higher $K_i$ value with propionate in the wild-type compared to the small $K_m$ of L-lactate suggests the hydroxyl group of the substrate is important for binding via H-bonding with protein. The transition state destabilization induced by the mutation and binding modeling suggests that the Tyr254 hydroxyl group stabilizes the Michaelis complex formation and transition state. Later on, the pH profile of wild-type and Tyr254Phe mutant investigated by Fitzpatrick’s group also showed the importance of neutral Tyr254 in the catalysis (58).
Figure 1.4 The active site of flavocytochrome b2 from baker’s yeast Saccharomyces cerevisiae (PDB code 1FCB).

The studies of Tyr129 in spinach glycolate oxidase also support the stabilization of the transition state by H-bonding between the substrate and hydroxyl group of Tyr129 (59). Oxalate, transition state analogue, can bind and inhibit glycolate oxidase wild-type (60). The loss of binding and inhibition in the Tyr249Phe enzyme suggest that the removal of hydroxyl group lose the interaction with transition state analogue. Tyr152 in lactate monoxygenase was studied by replacing with phenylalanine (61). The correlation of binding of oxalate and catalytic efficiency of wild-type and Tyr152Phe suggests the Tyr152 is involved in binding of transition state analogue oxalate. Moreover, the dissociation constant for sulfite is 100-fold higher in Tyr152Phe which is also observed in homologous mutation Tyr129Phe in glycolate oxidase and Tyr254Phe in flavocytochrome b2. In porcine D-amino acid oxidase, since the characteristics of sulfite binding and N5 sulfite adduct in tyrosine mutants and the chemical modification studies of Tyr228Phe are similar to the Tyr129Phe in glycolate oxidase and Tyr254Phe in flavocytochrome b2 (62), the similar role of Tyr228 was proposed.

Tyr201Phe in p-hydroxybenzoate hydroxylase was investigated with modeling and inhibition studies (63, 64). C(4a)-flavinhydroperoxide was used as a model to describe the transition state. According to the calculation of activation barrier, Tyr201 stabilizes both ground state and transition state via H-bonding with substrate. The failure to stabilize the substrate analogue, 4-
fluorobenzoate, and inhibitor, 4-hydroxycinnamate in the Tyr201Phe mutant suggests Tyr201 is important to activate substrate by forming H-bond with the hydroxyl group of substrate.

Based on sequence alignment and crystal structure studies, Tyr132 in human glycolate oxidase (65), Tyr146 in L-lactate oxidase (66), Tyr131 in mandelate dehydrogenase (55) are proposed to stabilize the transition state.

The role of Tyr143 in flavocytochrome b2 has also been probed with crystal structure, site-directed mutagenesis, stopped-flow, steady state and kinetic isotope effects (67). The substrate kinetic isotope effect on \( k_{\text{red}} \) of Tyr143Phe was smaller and the \( K_d \), which has been calculated based on the \( K_m \) and substrate kinetic isotope effect on \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \), was 4-5 fold higher than the wild-type. Based on the calculated free energy, these data suggest that the Tyr143 stabilizes the Michaelis complexes via H-bonding with the carboxylate group of the substrate.

**1.3.2.2 Component of Proton Relay Systems**

Hydroxyl protein groups can also be part of a proton relay system in a H-bonding network. The single and double mutants of Ser127 and Ser16 in chorismate synthase show the importance of these two serine residues in proton relay among the FMN, His106 and ligand EPSP (68). These two residues are conserved in bacterial, fungal, plant and protozoan chorismate synthase. The unchanged binding affinity to the FMN in all these mutants and slightly increased binding affinity only in the double mutants show the enzymes fold properly. However, the decreased activity in single mutant and no activity in the double mutants, combined with the crystal structure (69) shows that the H-bonding network is disrupted in the double mutants.

The roles of Ser175 and Thr178 in class 2 dihydroorotate dehydrogenase were studied by crystal structure, molecular dynamic simulation, site-directed mutagenesis, redox potential, and pH profile of flavin reduction (70-73). The data showed that the Ser175Ala, Thr178Ser,
Thr178Ala and Thr178Val mutant enzymes were folded properly and the active site environment was little affected based on the absorbance spectra and circular dichroism spectra. The decreased redox potential in the Ser175Ala and Thr178Ala mutants, about 40 mV, was due to hydrogen bond disruption. The slower hydride transfer in the mutants together with simulation shows the proton transfer from substrate to Ser175, through water to Thr178 to deprotonate the substrate, as shown in Figure 1.5. Thr48 in monomeric sacrosine oxidase (74) and Thr66 in heterotetrameric sacrosine oxidase (75) were also proposed to be a part of a proton relay system from the crystal structure. However, thus far the functions of these residues have not yet kinetically studied.

![Figure 1.5](image)

**Figure 1.5** The proton relay in class 2 dihydroorotate dehydrogenase (PDB code 1f78).

### 1.3.2.3 Aligning the Substrate into the Right Position for Catalysis

The role of Tyr223 in yeast D-amino acid oxidase has been probed by studying the wild-type, Tyr223Phe and Tyr223Ser variants (76). The spectra, ligand binding and redox potential studies suggest that mutagenesis has no significant effect on redox potential and the binding pocket. The kinetic parameters for Tyr223Phe are similar to wild-type. In contrast, the $K_d$ in Tyr223Ser mutant is increased 60-fold and product release is 800-fold slower. These data suggest the side chain of Tyr223 is not important for catalysis. Probably the steric effect of the side chain as well
as H-bonding is important for aligning the substrate in the right position for catalysis. Tyr238 residue was also investigated by the same group (77). Rapid reaction studies of the enzymes show the rates of the Tyr238Phe and Tyr238Ser mutants are similar to wild-type, suggesting this residue is not responsible for acid/base catalysis. The binding of inhibitors and substrate specificity of the mutants are also not significant different from wild-type, therefore, the residue 238 is not important for the substrate binding. However, the simulation analysis of experiment traces with program A shows that the substrate binding and product release steps are slower in the mutant. The effect of pH on the ligand binding and the flapping of Tyr238 side chain in the crystal structure as well as the kinetic parameters obtained from Tyr238 mutants propose that Tyr238 acts as a gate to control the substrate and product exchange.

Tyr92 residue in aryl-alcohol oxidase was also proposed to have the similar role of Tyr238 base on the molecular docking and steady state kinetic studies of wild-type and Tyr92Ala/Phe mutants. The results show that there is no catalytic activity observed in Tyr92Ala mutant, however, the activity of Tyr92Phe is similar to the wild-type (78). The crystal structure of aryl-alcohol oxidase also suggests the aromatic side chain of Tyr92 act as a gate which controls the entry of the substrate to the active site (79).

Tyr317 in monomeric sacrosine oxidase was proposed to bind and activate substrate (80). The kinetic studies of Tyr317Phe show the $k_{\text{cat}}/K_m$ and $k_{\text{red}}$ values were 15-fold and 20-fold lower than wild-type, respectively. The pH profile of $k_{\text{red}}$ in Tyr317Phe with the similar $pK_a$ as in the wild-type rules out the possibility of Tyr317 as a base. The $K_d$ values of Tyr317Phe are pH dependent with a $pK_a$ value of 9.0 which is not observed in wild-type enzyme. Moreover, the $K_d$ values of Tyr317Phe with inhibitors are 14- and 21-fold increased compared to the wild-type.
Together, the Tyr317 residue plays an important role in binding the substrate for efficient catalysis.

Ser267 is not in the active site in nitroalkane oxidase. However, steady state studies of Ser267Ala show that $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values are significantly lower than wild-type with nitroethane, 1-nitrohexane or 1-nitrooctane as substrate. The $k_{\text{red}}$ was also 80-fold lower than wild-type enzyme (81). The crystal structure of Ser267Ala shows that the residue Asp402 which was proposed to abstract proton of the substrate was not in the right position, compared with wild-type enzyme (81). Therefore, the hydroxyl group of Ser267 plays a role in aligning the Asp402 to the right position via H-bonding.

The crystal structure of aklavinone-11-hydroxylase shows Tyr224 forms a H-bond with aklavinone (82). No catalytic activity was detected in Tyr224Phe variant. These data suggests that the Tyr224 plays an important role in catalysis and maybe position the substrate for catalysis. In 2-nitropropane dioxygenase, the role of Ser288 was proposed (83). Ser288Ala of cannot be expressed in *E. coli* (83). Based on the crystal structure, the side chain of Ser288 was proposed to form H-bond with the nitro group of 2-nitropropane and bring it to the active site (83).

### 1.3.2.4 Involvement in Binding Substrate

The hydroxyl protein group is also proposed to affect the substrate binding by H-bond with the substrate carboxylate group, such as Tyr24 in spinach glycolate oxidase (84), Tyr26 in human glycolate oxidase (65) and Ser212 in p-hydroxybenzoate hydroxylase (85). Steady state kinetic, rapid reaction and crystallography were used to study the wild-type spinach glycolate oxidase and Tyr24Phe mutants (84). The results show that the $K_m$ and $K_d$ of glycolate in the mutant are 10- and 17-time increased, respectively. However, the turnover number and flavin
reduction rate constant are only slightly affected. Substitution of Ser212 in p-hydroxybenzoate hydroxylase to alanine results the $K_d$ value 10-fold increase and no change in the catalytic activity (85). Moreover, there are two binding conformations in the Ser212Ala with alternate substrate, suggested by the two products formed with HPLC analysis (85). Based on the crystal structure, Tyr246 in glycine oxidase (86), Tyr40 in L-lactate oxidase (87) (66), Tyr249 in D-arginine dehydrogenase (88) and Tyr26 in modelate dehydrogenase (55) are also H-bond with the substrate carboxylate group and may play a similar role in substrate binding.

1.3.2.5 Acting as a Base or Acid

Ser175 in class 2 dihydroorotate dehydrogenase was proposed to be a weak base which abstracts the proton from C5 of DHO substrate (73, 89, 90). Substitution of serine to alanine or cysteine causes the activity to decrease 10,000- and 500-fold, respectively (89). The flavin reduction in Ser175Ala is increased while increasing in pH with no observable $pK_a$, which is different from wild-type enzyme with a $pK_a$ of 9.5 (69). The crystal structure (71) and studies of conserved residue Cys130 in class 1 dihydroorotate dehydrogenase (91) also suggests that Ser175 is a base in this enzyme.

In chromate reductase from *Thermus scotoductus* SA-01, the FMN is reduced by NADPH and a hydride from N5 of reduced FMN with a proton transfers to the substrate. Tyr177 forms H-bond with substrate and was proposed to be a proton donor (92). However, the phenylalanine mutant was studied and only results in 5-fold decreasing of the activity. In the absence of Tyr177, the other residue or water may act as a proton donor. For the sequence alignment, Tyr196 in old yellow enzyme was also proposed to be a proton donor.
Tyr429 in glucooligosaccharide oxidase acts as a general base to abstract the hydroxyl proton of substrate based on the crystal structure (93). The maximum activity at pH 10 also supports this proposal (94).

1.3.2.6 Contributing to the Affinity of the Flavin to Apoprotein

In human medium chain acyl-CoA dehydrogenase, the role of Thr136 was studied by substitution with serine, aspartate and leucine (95). The analyses of in vitro and prokaryotic expression products show that the Thr136Ser has the similar biogenesis and activity as the wild-type. By adding riboflavin and FAD to culture broth and buffers, the Thr136Asp can form a catalytically active tetramer. However, no activity can be rescued in the Thr136Leu. Based on the crystal structure, the hydroxyl group of the Thr136 forms a H-bond with flavin N1-C2 atoms (96). Altogether, the hydrophilic group of Thr136 was proposed to bind FAD. The important of Thr168 to bind FAD in this enzyme was also studied by Andresen’s group (97). The Thr168Ala was not well folded and it is presumed to have lost the ability to bind FAD.

In pig kidney D-amino acid oxidase, Thr317 in the N1-C2 position plays a role in the affinity of FAD (98). The alanine mutant has a lower affinity to FAD than in the wild-type. The 2-13C labeled FAD-NMR spectra of wild-type and Thr317 shows that Thr137 forms a H-bond with flavin C(2)=O. Based on the sequence alignment, Tyr338 in yeast D-amino acid oxidase (99), Thr317 in human D-amino acid oxidase (99) are conserved and proposed to have similar roles.

Ser99 porcine cytochrome b5 reductase is proposed to maintain the conformation of FAD by H-bonding to the phosphate group (100, 101). Absorbance spectra and denaturation studies were investigated in the Ser99Thr, Ser99Ala and Ser99Val mutants. Except for the Ser99Thr, the other two mutants result in obvious spectral and stability changes. Therefore, the hydroxyl group of Ser99 is important to maintain the conformation of FAD. Based on the sequence alignment,
Ser492 in NADPH-cytochrome p450 reductase, Thr98 in nitrate reductase, Ser133 in ferredoxin-NADP+ reductase, Ser84 in phthalate dioxygenase reductase and Ser76 in flavodoxin reductase are proposed to have the same role.

1.3.2.7 Maintaining the Protein Structure

Ser171 in nitroalkane oxidase was studied by substitution with alanine, valine and threonine (102). There is no significant difference in the mutant from the wild-type enzyme with steady state kinetic and kinetic isotope effect studies. However, the crystal structure shows the hydroxyl group of Ser171 forms a H-bond with N5 flavin. Therefore, Ser171 is believed to not be involved in catalysis but may be important for maintaining the protein structure.

Tyr65 in porcine cytochrome b5 reductase is close to 4’-hydroxyl of the ribityl moiety of the FAD and the hydroxyl group and is important in protein stability based on the studies of Tyr65Ala and Tyr65Phe mutants (100, 101). The absorbance spectra and CD spectrum of Tyr65Ala exhibit large changes instead of Tyr65Phe. Also, the fluorescence emission spectra of both mutants were different from the wild-type.

1.3.2.8 Other Functions

The role of Tyr169 in trimethylamine dehydrogenase has been investigated by site-directed mutagenesis, stopped-flow and the EPR spectroscope. Based on the crystal structure, Tyr169 is Van der Waals bonded to the flavin C(2)=O locus and H-bonded to His172 (103). In comparison to the EPR spectra of the wild-type with the mutant, the spin-interaction between flavin semiquinone and reduced 4Fe/4S center seen in the wild-type was not observed in the Tyr169Phe mutant, which suggest that Tyr169 residue plays an important role in mediating the spin-interaction (104).
The two tyrosine residues were found at the active site of human monoamine oxidase B which forms an “aromatic sandwich” with substrate analog, as shown in Figure 1.6 (105). The wild-type enzyme as well as Tyr435Phe, Tyr435His, Tyr435Leu and Tyr435Trp were studied with crystal structure and steady state kinetics (106). Crystal structures of mutants shows there is no significant difference from the wild-type, suggesting that the mutants are covalently linked to FAD and fold well as the wild-type. Analyzing the $k_{cat}/K_m$ values with the dipole-dipole interaction energy together proposed nucleophilic mechanism indicates that the dipolar effect of tyrosine side chains activate the substrate amine for catalysis by increasing its nucleophilicity.

![Figure 1.6](image.png)

**Figure 1.6** The “aromatic sandwich” in human MAO B (PDB code 1GOS).

### 1.4 Specific Goals

The dissertation aims to study the role of the serine proximal to the flavin C(4a)-N(5) atoms in choline oxidase and the kinetic mechanism of D-arginine dehydrogenase to gain insight in the mechanism for alcohol or amine oxidation.

Choline oxidase (E.C.1.1.3.17) from *A. globiformis* catalyzes the oxidation of choline to glycine betaine via two reductive half-reactions and two oxidative half-reactions. This reaction is of considerable interest for medical and biotechnological applications because the product,
glycine betaine, is a compatible solute (107-109). Experimental data have shown that cells with the codA gene encoding choline oxidase can grow well in the presence of 0.4 M NaCl. However, cells without the codA gene cannot survive, as shown in Figure 1.8 (110). In the wild-type enzyme, catalysis is triggered by the kinetically fast abstraction of the substrate hydroxyl group by a catalytic base, followed by the transfer of a hydride ion from the α-carbon of the substrate to the N5 atom of flavin, as shown in Scheme 1.8. Several residues in the active site have been studied to contribute the catalysis of oxidation of choline (Figure 1.9). His99, covalently linked to FAD, is important to align FAD in the optimal position to accept hydride ion in the oxidation of choline (111). Glu312 is important for substrate binding via electrostatic interaction with the trimethylammonium group of choline (112, 113). Val464 provides a non-polar site for oxygen access into the active site (114, 115). His351 and His466 are proposed to stabilize the alkoide species (116, 117). Asn510 is important for the flavinylation reaction in which FAD is covalently attached to the protein moiety in choline oxidase (118).

**Figure 1.7** Left panel, glycine betaine as an osmoprotectant for *S. meliloti*. Right panel, Growth under 0.4 M NaCl in control cells *Synechococcus* sp. PCC 7942 (PAM) and transformed cells (PAMCOD) in which the codA gene (for choline oxidase) was introduced. Modified from ref. (110), taken without permission.
a) Proton abstraction:

b) Hydride transfer:

Scheme 1.8 Hydride Transfer Mechanism for the Oxidation of Choline to Betaine Aldehyde Catalyzed by Choline Oxidase

Figure 1.8 Active site of choline oxidase (PDB# 2j bv)

Based on the crystal structure of choline oxidase wild-type, a serine residue at position 101, which is less than 4 Å from the flavin N5 atom and within hydrogen bonding distance to the
ligand DMSO, was investigated to probe the role of the hydroxyl group of Ser101 in the reaction catalyzed by the enzyme. To elucidate this, the variants in which the serine was replaced with alanine, threonine, cysteine or valine were prepared and purified. The effects of hydroxyl group at position 101 on the catalytic efficiency of choline oxidase in both the reductive and the oxidative half-reactions were studied with crystallographic and mechanistic approaches. The details will be described in Chapters 2, 3 and 4.

D-arginine dehydrogenase (DADH) from *Pseudomonas aeruginosa* catalyzes the oxidation of D-amino acids to the corresponding imino acids, which are non-enzymatically hydrolyzed in solution to α-keto acids and ammonia, as shown in Scheme 1.9. D-amino acids are important for bacteria as fundamental elements of the bacterial cell wall peptidoglycan layer (119). It was reported that D-leucine, D-methionine, D-tyrosine and D-tryptophan can regulate disassembly of bacterial biofilms (120). D-aspartate was found in the brain and other tissues of mammals and plays a role in regulating the development of these organs (121). D-serine may act as an endogenous agonist of the N-methyl-D-aspartate receptor in the rat brain (122). In *P. aeruginosa*, D-arginine can be the sole source of carbon and nitrogen (123). Dr. Lu’s group, Biology department in Georgia State University, found that the racemization of D- to L-arginine conversion is provided by a two-enzyme-coupled system: DADH and L-arginine dehydrogenase. The products of DADH, keto acid and ammonia, is catalyzed by L-arginine dehydrogenase to L-arginine. The discovery of D-arginine dehydrogenase suggests the existence of the fourth arginine catabolic pathway in *P. aeruginosa* (124).

\[
\begin{align*}
\text{unknown } e^- \text{ acceptor} & \\
\text{R} & \text{H} \quad \text{FAD} \quad \text{FADH}_2 \\
\text{NH}_3^+ & \quad \rightarrow \\
\text{R} & \text{COO}\text{NH}_2^+ \quad + \text{H}_2\text{O} \\
\text{R} & \text{COO}\text{NH}_2^+ \quad + \text{NH}_4^+ \\
\end{align*}
\]

Scheme 1.9 Mechanism Proposed for Oxidation of D-Amino Acids by DADH.
The mechanism for primary amine oxidation catalyzed by flavin-dependent enzymes has been debated for several decades. In addition to the hydride transfer mechanism, several mechanisms were also proposed for flavoenzyme reductive half-reactions. In order to understand the mechanism of carbon-nitrogen oxidation, the DADH can be a candidate to study. The studies will be discussed together with the structural information in chapters 5, 6 and 7.

1.5 References


66. Umema, Y., Yorita, K., Matsuoka, T., Kita, A., Fukui, K., and Morimoto, Y. (2006) The crystal structure of L-lactate oxidase from Aerococcus viridans at 2.1A resolution reveals


VAAGL hydrophobic stretch located at the si-face of the flavin ring, *Protein Sci* 15, 2708-2717.


Chapter 2

Structural and Kinetic Studies on the Ser101Ala Variant of Choline Oxidase: Catalysis by Compromise

(This chapter has been published verbatim in Finneghan, S., Yuan, H., Wang, Y., Orville, A.M., and Gadda, G., (2010), Archives of Biochemistry and Biophysics 501, 207-213. The author’s contribution to this chapter involved the preparation and purification of the mutant enzyme, the determination of the steady state kinetic parameters and the reductive half-reaction kinetic parameters.)

2.1 Abstract

The oxidation of choline catalyzed by choline oxidase includes two reductive half-reactions where FAD is reduced by the alcohol substrate and by an aldehyde intermediate transiently formed in the reaction. Each reductive half-reaction is followed by an oxidative half-reaction where the reduced flavin is oxidized by molecular oxygen. In the present study, we have used site-directed mutagenesis to prepare the Ser101Ala mutant of choline oxidase and have investigated the impact of this mutation on the structural and kinetic properties of the enzyme. The X-ray crystallographic structure of the Ser101Ala enzyme resolved to 2.2 Å indicates that the only differences between the mutant and wild-type enzymes are the lack of a hydroxyl group on residue 101 and a more planar configuration of the flavin isoalloxazine ring with respect to that of the wild-type enzyme. Steady state and rapid reaction kinetics established that replacement of Ser101 with alanine yields a mutant enzyme with increased efficiencies in the oxidative half-reactions and decreased efficiencies in the reductive half-reactions. This is accompanied by a significant decrease in the overall rate of turnover with choline. Thus, this mutation has revealed the importance of a specific residue for the optimization of the overall
turnover of choline oxidase, which requires fine-tuning of four consecutive half-reactions for the conversion of an alcohol to a carboxylic acid.

2.2 Introduction

The reaction of choline oxidation catalyzed by choline oxidase (E.C. 1.1.3.17; choline-oxygen 1-oxidoreductase) has been extensively characterized (see (1) for a recent review) due to its relevance for genetically engineering crops with increased tolerance to environmental stress and the potential for designing therapeutic agents against pathogenic bacteria (2-5). In brief, the reaction includes two reductive half-reactions where the FAD cofactor is reduced by the alcohol substrate and by an enzyme-associated aldehyde intermediate (Scheme 2.1) (6). Each reductive half-reaction is followed by an oxidative half-reaction where the reduced FAD cofactor is oxidized by molecular oxygen with formation of hydrogen peroxide (6). In the wild-type enzyme, the first reductive half-reaction is initiated by a kinetically fast abstraction of the hydroxyl proton of choline, which results in the formation of a transient alkoxide intermediate (6). This is followed by a rate-limiting hydride ion transfer from the α-carbon of the alkoxide intermediate to the N(5) atom of the flavin resulting in the oxidation of choline to betaine aldehyde and reduction of the flavin (6). Betaine aldehyde is subsequently hydrated in the active site of the enzyme to form gem-diol choline (7, 8). In the second reductive half-reaction, the gem-diol choline is oxidized to the product, glycine betaine. In the oxidative half-reactions, the reduced flavin reacts with oxygen by transferring two electrons to oxygen to form oxidized flavin and hydrogen peroxide (9). These reactions occur when the organic product of the enzymatic reaction is bound at the active site, rather than after its release to the solvent (6, 9). Maximal overall enzymatic turnover is therefore attained through a fine balancing of the
requirements that are necessary for each of the half-reactions to go forward efficiently without negatively impacting any of the other half-reactions.

By using site-directed mutagenesis the mechanistic roles of several functional groups in the active site of the choline oxidase from *Arthrobacter globiformis* have been elucidated. His99, which is the site of covalent attachment of the flavin to the protein moiety, is important for the optimal positioning of FAD in the enzyme-alkoxide complex, thereby facilitating the environmentally assisted transfer of the hydride ion in the oxidation of choline (10). Glu312 is the main site of substrate anchoring through electrostatic interaction with the trimethylammonium group of choline, playing a role for the optimal positioning of the substrate for efficient catalysis (11, 12). His351 is important for the binding and positioning of the substrate for efficient hydride ion transfer, for stabilization of the transition state developed during choline oxidation, and for fine-tuning the polarity of the active site (13). Val464 participates primarily in the oxidative half-reaction where the reduced flavin is oxidized by oxygen, with minimal effects on the reaction of choline oxidation (14, 15). Finally, His466 modulates the electrophilicity of the enzyme-bound flavin, the polarity of the active site and contributes to the stabilization of the transition state for the oxidation of choline to betaine aldehyde (16, 17). Interestingly, none of the three active site histidines of choline oxidase (i.e., His99, His351, and His466) provides electrostatic stabilization of either the superoxide anion intermediate or the transition state that are formed in the reaction of the reduced flavin with oxygen (10, 13, 16). In contrast, such an electrostatic stabilization is exerted by the positive charge of the enzyme-bound organic molecule undergoing oxidation in the reaction catalyzed by choline oxidase, as suggested by mechanistic studies with a substrate analog devoid of positive charge (18, 19).
In the crystal structure of wild-type choline oxidase, the side chain of Ser101 is less than 4 Å from the N(5) atom of FAD and within hydrogen bonding distance of the oxygen atom of DMSO, a ligand that was used in the crystallization of the enzyme (11). This suggests that Ser101 may be actively involved in the oxidation of choline catalyzed by the enzyme. Here, we report the expression, purification, as well as the crystallographic and kinetic characterizations of the Ser101Ala variant of the enzyme. The results showed that replacement of Ser101 with alanine increases the apparent rates for the oxidative half-reactions by three-fold, while decreasing those for the reductive half-reactions and the overall turnover of the enzyme by ten-fold. Thus, while the hydroxyl side chain of Ser101 is not essential for catalysis, it is important for the optimization of the enzymatic turnover of choline oxidase.

![Scheme 2.1 The Steady State Kinetic Mechanism of Choline Oxidation Catalyzed by Choline Oxidase.](image)

**Scheme 2.1** The Steady State Kinetic Mechanism of Choline Oxidation Catalyzed by Choline Oxidase. E, enzyme; FADox, oxidized flavin; CH, choline; FADred, reduced flavin; BA, betaine aldehyde; GB, glycine betaine.

2.3 **Experimental Procedures**

The mutant gene for the Ser101Ala enzyme was prepared using the QuikChange™ Site-Directed Mutagenesis kit following the manufacturer’s instructions in the presence of 2%
DMSO, as previously described (17, 20). The pET/codA mg plasmid harboring the wild-type gene was used as template for mutagenesis (20). Upon mutagenesis, the entire mutant gene (pET/codA mg-Ser101Ala) was sequenced at the DNA Core Facility of Georgia State University to confirm the presence of the desired mutation. As an expression host, competent *Escherichia coli* Rosetta(DE3)pLysS cells were transformed with the mutant plasmid by electroporation. The mutant enzyme was expressed and the resulting enzyme was purified to homogeneity as previously described for wild-type choline oxidase (17, 18, 20).

Crystals of the Ser101Ala enzyme were grown by the hanging-drop vapor-diffusion method at room temperature. Purified Ser101Ala (2 μL) at a concentration of 5 mg mL⁻¹ was mixed with 2 μL from a 500 μL reservoir solution consisting of 80 mM sodium cacodylate, 20% v/v PEG6000, 20% v/v glycerol, 150 mM Mg-acetate at pH 6.0. Single crystals were transferred into a cryoprotectant consisting of reservoir solution containing 25% (v/v) glycerol and allowed to soak for approximately 2 min prior to flash-freezing in liquid nitrogen for data collection at Beamline 12B of the National Synchrotron Light Source at Brookhaven National Laboratory, NY. The data were integrated, scaled, and merged using the HKL2000 package (21). The structures were solved by molecular replacement with Phaser (22) using the structure of wild-type choline oxidase (2JBV from the Protein Data Bank) as the starting model (23). Refinement was carried out using Refmac5 (24) in CCP4 (25) and manual adjustment used the molecular graphics program COOT (26). Structural figures were made with PyMol software (27).

Steady state kinetic parameters were measured with the method of the initial rates (28) at varying concentrations of both choline, or betaine aldehyde, and oxygen in 50 mM sodium pyrophosphate, pH 10.0, at 25 °C. Kinetic assays were performed at pH 10.0 because at this pH value the kinetic parameters $k_{cat}$ and $k_{cat}/K_{m}$ of choline oxidase are maximal and independent of
pH (9). Initial rates were determined by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument Ltd.) at 25 °C. The assay reaction mixture was equilibrated at the desired concentration of oxygen by sparging the appropriate O₂/N₂ gas mixture for 10 min before the reaction was started with the addition of the enzyme. The initial rates measured with choline were fit to eq 1, which describes a sequential steady state kinetic mechanism in which \( K_{choline} \) and \( K_{oxygen} \) are the Michaelis constants for choline and oxygen and \( k_{cat} \) is the overall turnover number of the enzyme (e) when saturated with both substrates. The initial rates measured with betaine aldehyde were fit to eq 2, which describes a sequential steady state kinetic mechanism of the type described by eq 1 where \( K_{aldehyde} \ll K_{oxygen}K_{ia}. \)

\[
\frac{v}{e} = \frac{k_{cat}[choline][oxygen]}{K_{choline}[oxygen]+K_{oxygen}[choline]+[choline][oxygen]+K_{ia}K_{oxygen}}
\]

(1)

\[
\frac{v}{e} = \frac{k_{cat}[betaine-aldehyde][oxygen]}{K_{ia}K_{oxygen}+K_{oxygen}[betaine-aldehyde]+[betaine-aldehyde][oxygen]}
\]

(2)

The reductive half-reaction with betaine aldehyde was carried out by using a Hi-Tech SF-61 stopped-flow spectrophotometer at 25 °C and pH 10.0. The rate constants for flavin reduction were measured by monitoring the decrease in absorbance at 452 nm that results from the anaerobic mixing of the enzyme and betaine aldehyde, as previously described for the wild-type enzyme (6). Glucose (5 mM) and glucose oxidase (0.5 µM) were added to the substrate and enzyme solutions to scavenge possible trace amounts of oxygen. The Ser101Ala enzyme was mixed anaerobically with an equal volume of betaine aldehyde, obtaining reaction mixtures with 10 µM enzyme and 0.2-5 mM betaine aldehyde. At each concentration of the substrate, the rate constants for flavin reduction were recorded in triplicate, with measurements usually differing by ≤5 %. Stopped-flow traces were fit to eq 3, which describes a single exponential process where
$k_{\text{obs}}$ is the observed first-order rate constant for flavin reduction, $A$ is the value of absorbance at 452 nm at time $t$, $B$ is the amplitude of the absorbance change, and $C$ is an offset value that accounts for the non-zero absorbance value at infinite time. Kinetic parameters for the reductive half-reaction were determined by using eq 4, where $k_{\text{obs}}$ is the observed first-order rate constant for the reduction of the enzyme-bound flavin at any given concentration of substrate, $k_{\text{red}}$ is the limiting first-order rate constant for flavin reduction at saturated substrate concentration, and $K_d^{\text{app}}$ is the macroscopic dissociation constant for binding of the substrate to the enzyme.

\begin{align}
A &= B \exp(-k_{\text{obs}}t) + C \\
k_{\text{obs}} &= \frac{k_{\text{red}}A}{K_d + A}
\end{align}

2.4 Results

The Ser101Ala enzyme was expressed and purified to high yields by using the same protocol previously used for the wild-type enzyme (11, 20). As in the case of wild-type choline oxidase, the enzyme-bound flavin was present as a mixture of oxidized and anionic flavosemiquinone throughout the purification procedure, with the fully oxidized enzyme obtained after extensive dialysis at pH 6.0 (9, 20). The purified Ser101Ala enzyme showed absorbance bands centered at 373 nm ($\varepsilon = 8.9$ mM$^{-1}$cm$^{-1}$) and 452 nm ($\varepsilon = 9.5$ mM$^{-1}$cm$^{-1}$) at pH 8.0, compared to 367 nm ($\varepsilon = 10.0$ mM$^{-1}$cm$^{-1}$) and 455 nm ($\varepsilon = 11.4$ mM$^{-1}$cm$^{-1}$) for the wild-type enzyme (9), consistent with an altered flavin microenvironment in the mutant enzyme. The enzymatic activity of the Ser101Ala enzyme was 3.3 s$^{-1}$ with saturated choline (i.e., 10 mM) at pH 7.0 and 25 °C, compared to 15 s$^{-1}$ for the wild-type (11), suggesting that Ser101 is important, but not essential, for catalysis.
The Ser101Ala enzyme crystallized in the primitive monoclinic space group P2\textsubscript{1} with eight subunits assembled as four homodimers in the asymmetric unit. X-ray data to 2.2 Å were used to refine the structure of the Ser101Ala enzyme to a final R-factor of 22.8% (Table 2.1). The final model consisted of residues 1 to 530\textsuperscript{1}, FAD, and acetate from the crystallization solution in the active site of the enzyme. The acetate ligand in the active site of the Ser101Ala enzyme had the same orientation and positioning in the eight subunits that crystallized; its oxygen atoms are 3.8 Å from both the flavin N(5) atom and the N(3) atom of His351 (Figure 2.1C). When the homodimers of the Ser101Ala enzyme were superimposed on those of the wild-type enzyme the average rmsd values were 0.41 Å for 527 equivalent C\textalpha atoms in each chain, indicating no significant structural differences between the backbone atoms of the two enzymes. The active sites of the two structures are compared in Figure 2.1, showing essentially no differences in the relative location of the flavin and the active site residues in the two enzymes, although there are differences in the conformation of the flavin. Interestingly, although the replacement of the side chain on residue 101 from serine to alanine resulted in loss of a hydrogen bond with His351, all of the active site residues are located in the same positions and orientations in the mutant and wild-type enzymes. Moreover, the electron density maps clearly indicate that FAD in the Ser101Ala enzyme is covalently linked to the His99 N\textepsilon\textsubscript{2} atom as seen in the wild-type (11).

A notable difference between the wild-type and the Ser101Ala enzymes is the isoalloxazine moiety of the FAD cofactor in the variant enzyme being more planar than that of the wild-type. In the latter enzyme, the C(4a) atom was shown to be in an \textit{sp\textsuperscript{3}} hybridization due to the presence of an oxygen adduct that is not observed in the Ser101Ala variant enzyme resulting in the C(4a)

---

\textsuperscript{1} In the 3D structure of the Ser101Ala enzyme reported here (3NNE), the last 16-18 amino acids at the C-terminus of different subunits are not observable. Similarly, the wild-type enzyme was reported with coordinates for the first 528 residues and no ordered density for the last 18 amino acid residues at the C-terminus (2JBV). A third structure of a choline oxidase variant recently reported also has coordinates for the first 530 residues, but not for the last 16 residues at the C-terminus (3LJP). This is most likely due to the C-terminal end of the protein being highly flexible.
atom being $sp^2$ hybridized \((11, 29)\). In the Ser101Ala enzyme the isalloxazine ring system showed a slight bend between the two planes containing the benzene and pyrimidine moieties defining an 8° angle along the N(5)-N(10) axis (Figure 2.1B). A similar bend was recently observed in the structure of another active site mutant form of choline oxidase where Val464 is replaced with alanine, for which the C(4a) atom of FAD was also shown to be $sp^2$ hybridized \((14)\). In summary, the major differences observable in the structures of the Ser101Ala and wild-type enzymes are the hybridization of the flavin C(4a) atom and the loss of the hydroxyl group of Ser101.

The steady state kinetic parameters with choline or betaine aldehyde as substrate for the Ser101Ala enzyme were determined at varying concentrations of oxygen, by measuring the initial rates of oxygen consumption with a Clark-type oxygen electrode at pH 10.0 and 25 °C. As for the case previously reported for the wild-type enzyme \((8, 9)\), the best fits of the data were obtained with eq 1 for choline and eq 2 for betaine aldehyde (Figure 2.2). As summarized in Table 2.2, the Ser101Ala enzyme showed between three-fold and four-fold increases in the second-order rate constants for oxygen \((k_{cat}/K_{oxygen})\) with choline and betaine aldehyde. In contrast, a ten-fold decrease in both the second-order rate constant for the capture of choline \((k_{cat}/K_{choline})\) and the overall turnover number of the enzyme with choline as substrate \((k_{cat})\) were observed. Since the \(k_{cat}/K_{betaine\text{ aldehyde}}\) value could not be determined by using a steady state kinetic approach due to \(K_{aldehyde} \ll K_{oxygen}K_{ia}\), the reductive half-reaction with betaine aldehyde was investigated by anaerobically mixing the enzyme and the substrate in a stopped-flow spectrophotometer at pH 10.0 and 25 °C. As previously reported for the wild-type enzyme \((6)\), the absorbance at 452 nm of the Ser101Ala enzyme decreased in a single exponential manner upon mixing with betaine aldehyde (Figure 2.3). A plot of the \(k_{obs}\) value for flavin reduction as a
function of the concentration of betaine aldehyde followed saturation kinetics, allowing the
determination of the limiting rate constant for flavin reduction at saturated substrate ($k_{\text{red}}$) and the
macroscopic equilibrium constant for the formation of the enzyme-betaine aldehyde complex
($K_{d}$). A second-order rate constant for the capture of betaine aldehyde to yield species
committed to catalysis ($k_{\text{red}}/K_{d}$) of 52,000 M$^{-1}$s$^{-1}$ was calculated from the stopped-flow kinetic
data (Table 2.2), which was six-fold lower than the value of 320,000 M$^{-1}$s$^{-1}$ previously
determined for the wild-type enzyme (6). Thus, replacement of Ser101 with alanine results in
lowered second-order rate constants for both the reductive half-reactions, but increased second-
order rate constants for both the oxidative half-reactions catalyzed by the enzyme.

Table 2.1 Crystallographic Data Collection and Refinement Statistics.

<table>
<thead>
<tr>
<th></th>
<th>P2$_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>P2$_1$</td>
</tr>
<tr>
<td><strong>Unit cell dimensions: (Å)</strong></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>69.26</td>
</tr>
<tr>
<td>B</td>
<td>346.03</td>
</tr>
<tr>
<td>C</td>
<td>105.92</td>
</tr>
<tr>
<td>$\beta$</td>
<td>94.33</td>
</tr>
<tr>
<td><strong>Unique reflections</strong></td>
<td>150,964</td>
</tr>
<tr>
<td><strong>$R_{\text{merge}}$ (%)</strong></td>
<td>11.1(25.8)$^a$</td>
</tr>
<tr>
<td><strong>$I/\sigma(I)$</strong></td>
<td>14 (3.2)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>90.4 (50.3)</td>
</tr>
<tr>
<td><strong>Data range for refinement (Å)</strong></td>
<td>10-2.47</td>
</tr>
<tr>
<td><strong>$R_{\text{cryst}}$ (%)</strong></td>
<td>22.8</td>
</tr>
<tr>
<td><strong>$R_{\text{free}}$ (%)</strong></td>
<td>29.2</td>
</tr>
<tr>
<td><strong>No. of solvent atoms</strong></td>
<td>333</td>
</tr>
<tr>
<td><strong>(total occupancies)</strong></td>
<td>(207.3)</td>
</tr>
<tr>
<td><strong>RMS deviation from ideality</strong></td>
<td></td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.014</td>
</tr>
<tr>
<td>Angle distance (Å)</td>
<td>0.021</td>
</tr>
<tr>
<td><strong>Average B-factors (Å$^2$)</strong></td>
<td></td>
</tr>
<tr>
<td>Main-chain atoms</td>
<td>26.4</td>
</tr>
<tr>
<td>Side-chain atoms</td>
<td>27.2</td>
</tr>
<tr>
<td>FAD</td>
<td>18.5</td>
</tr>
<tr>
<td>Solvent</td>
<td>20.3</td>
</tr>
</tbody>
</table>

*a* Values in parentheses are given for the highest resolution shell

\[ R_{\text{cryst}} = \frac{\sum |F_{\text{obs}} - F_{\text{cal}}|} {\sum F_{\text{obs}}}. \]

\[ R_{\text{free}} = \frac{\sum_{\text{test}} (|F_{\text{obs}} - |F_{\text{cal}}|)^2} {\sum_{\text{test}} |F_{\text{obs}}|^2}. \]
Figure 2.1 Comparison of crystal structures of the Ser101Ala and wild-type enzymes of choline oxidase. The residues with carbons in green represent the Ser101Ala structure, whereas the carbon atoms for the wild-type enzyme are in gray. Panel A shows the interactions at the Ser101Ala mutation site. Hydrogen bonds only present in the wild-type enzyme are colored red, those only in the Ser101Ala enzyme are colored green, and those observed in both enzymes are colored cyan. Panel B illustrates the superposition of the FAD isalloxazine ring from the wild-type (grey) with the C(4a)-oxygen adduct (red) and the Ser101Ala enzymes (colored by element type) of choline oxidase. Note that the isalloxazine ring of the Ser101Ala enzyme is more planar than that of the wild-type structure. Panel C shows the omit-Fourier density map with contour level at 2 for the active site of the Ser101Ala structure. Panel D shows a comparison of the active sites of wild-type and Ser101Ala choline oxidase. The wild-type enzyme structure is from pdb file 2jby (11).
Figure 2.2 Anaerobic Double reciprocal plots of Ser101Ala catalyzed oxidation of choline and betaine aldehyde. Choline oxidase activity was measured at varying concentrations of both choline and oxygen or betaine aldehyde and oxygen in 100 mM sodium pyrophosphate, pH 10.0, at 25 °C. Panel A, e/vo as a function of the inverse choline concentration determined at several fixed concentrations oxygen: (●) 0.033 mM; (○) 0.068 mM; (■) 0.172 mM; (□) 0.344 mM. Panel B, e/vo as a function of the inverse oxygen concentration determined at several fixed concentrations choline: (●) 0.1 mM; (○) 0.2 mM; (■) 0.5 mM; (□) 2 mM; (▲) 5 mM. Panel C, e/vo as a function of the inverse betaine aldehyde concentration determined at several fixed concentrations oxygen: (●) 0.16 mM; (○) 0.23 mM; (■) 0.36 mM; (□) 0.47 mM. Panel D, e/vo as a function of the inverse oxygen concentration determined at several fixed concentrations betaine aldehyde: (●) 0.5 mM; (○) 2 mM; (■) 5 mM; (□) 10 mM; (▲) 40 mM.
Figure 2.3 Reductive half-reaction of the Ser101Ala enzyme with betaine aldehyde. Panel A shows the reduction traces with 0.1 mM (black), 0.2 mM (blue), 0.3 mM (red), 2.5 mM (green) and 5 mM (fuscia) betaine aldehyde. All traces were fit to eq 3. Time indicated is after the end of flow, i.e., 2.2 ms. For clarity, one experimental point every 5 is shown (vertical lines). Panel B shows the observed rate constants for flavin reduction as a function of the concentration of betaine aldehyde. Data were fit to eq 4. Buffer used was 50 mM sodium pyrophosphate, pH 10.0.
Table 2.2 Comparison of the Kinetic Parameters of Ser101Ala and Wild-Type Choline Oxidase at pH10.0.

<table>
<thead>
<tr>
<th>substrate</th>
<th>kinetic parameters</th>
<th>definition(^c)</th>
<th>Ser101Ala</th>
<th>wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>choline</td>
<td>(k_{\text{cat}}, \text{s}^{-1})</td>
<td>(\frac{k_3 k_7}{k_3 + k_7})(^d)</td>
<td>6.7 ± 0.1</td>
<td>60 ± 1</td>
</tr>
<tr>
<td></td>
<td>(k_{\text{cat}}/K_m, \text{M}^{-1}\text{s}^{-1})</td>
<td>(\frac{k_1 k_3}{k_2 + k_3})</td>
<td>25,600 ± 2000</td>
<td>237,000 ± 9000</td>
</tr>
<tr>
<td></td>
<td>(k_{\text{cat}}/K_{\text{oxygen}}, \text{M}^{-1}\text{s}^{-1})</td>
<td>(\frac{k_5 k_9}{k_5 + k_9})</td>
<td>261,000 ± 6500</td>
<td>86,400 ± 3600</td>
</tr>
<tr>
<td></td>
<td>(K_m, \text{mM})</td>
<td>(\frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)})</td>
<td>0.26 ± 0.02</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(K_{\text{oxygen}}, \text{mM})</td>
<td>(\frac{k_7 k_9(k_5 + k_9)}{k_7 k_9(k_3 + k_7)})</td>
<td>0.026 ± 0.001</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(K_{\text{dox}}, \text{mM})</td>
<td>(\frac{k_7 k_9}{k_1 k_3(k_5 + k_9)})</td>
<td>3.6 ± 0.1</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>betaine aldehyde</td>
<td>(k_{\text{cat}}, \text{s}^{-1})</td>
<td>(k_7)</td>
<td>43 ± 3</td>
<td>133 ± 4</td>
</tr>
<tr>
<td></td>
<td>(k_{\text{cat}}/K_{\text{oxygen}}, \text{M}^{-1}\text{s}^{-1})</td>
<td>(k_9)</td>
<td>203,400 ± 33,400</td>
<td>53,400 ± 1600</td>
</tr>
<tr>
<td></td>
<td>(K_{\text{oxygen}}, \text{mM})</td>
<td>(k_7/k_9)</td>
<td>0.2 ± 0.03</td>
<td>2.5 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(k_{\text{red}}, \text{s}^{-1})</td>
<td>(k_7)</td>
<td>47 ± 2</td>
<td>135 ± 4</td>
</tr>
<tr>
<td></td>
<td>(K_d, \text{mM})</td>
<td>-(^e)</td>
<td>0.9 ± 0.1</td>
<td>0.45 ± 0.1</td>
</tr>
</tbody>
</table>

\(^a\) Conditions: 50 mM sodium pyrophosphate, pH 10.0, at 25 °C. \(^b\) From ref (6). \(^c\) Kinetic data are readily accounted for with the kinetic mechanism of Scheme 2.1 [6]. \(^d\) While \(k_7 >> k_3\), \(k_{\text{cat}}\) is equal to \(k_3\), \(K_m\) is equal to \((k_2 + k_3)/k_1\) and \(K_{\text{oxygen}}\) is equal to \(k_3(k_5 + k_9)/(k_5 k_9)\), as for the case of the Ser101Ala enzyme. \(^e\) Not applicable to the mechanism of Scheme 2.1.

2.5 Discussion

In choline oxidase, each of the four half-reactions in which the flavin is reduced by the organic molecules and oxidized by molecular oxygen is adjusted to achieve the most efficient overall turnover of the enzyme rather than maximal efficiencies. This conclusion stems from the comparison of the steady state and rapid reaction kinetic data of the Ser101Ala and the wild-type enzymes. Indeed, in spite of increased efficiencies in the oxidative half-reactions catalyzed by the enzyme lacking the hydroxyl group on residue 101, there is a ten-fold decrease in the overall
turnover of the enzyme with choline that is imputable to decreased efficiencies in the reductive half-reactions catalyzed by the enzyme. In the wild-type enzyme, Ser101 is on the si-face of the isoalloxazine moiety of the flavin with its hydroxyl group being 4 Å away from the flavin N(5) atom. Such a geometric arrangement of residue 101, as well as of all of the other residues in the active site of choline oxidase, is maintained in the crystallographic structure of the Ser101Ala enzyme, which clearly lacks the hydroxyl group. Thus, the differences seen in the kinetic properties of the mutant and wild-type enzymes are solely due to the lack of the hydroxyl group on residue 101, rather than originating from other geometric or steric factors.

Replacement of Ser101 with alanine results in decreased efficiencies in the reductive half-reactions with betaine aldehyde and choline. Evidence for this conclusion comes from the comparison of the steady state and rapid reaction kinetic data determined with the Ser101Ala and the wild-type enzymes presented here. Indeed, the second-order rate constants for the capture of choline ($k_{\text{cat}}/K_{\text{choline}}$) and betaine aldehyde ($k_{\text{red}}/K_d$) to yield enzyme-substrate complexes that are committed to flavin reduction were ten- and six-fold lower in the enzyme lacking the hydroxyl group on residue 101. In the wild-type enzyme, the chemical steps of flavin reduction with choline and the aldehyde intermediate are fully rate-limiting in both reductive half-reactions and are the sole contributors to the overall rate of turnover of the enzyme (6). Moreover, the rate for the overall turnover with betaine aldehyde as substrate is limited solely by the chemical step of flavin reduction, with a rate constant of 135 s$^{-1}$ (6). The latter conclusion also applies to the Ser101Ala enzyme, since the rate constant for flavin reduction with betaine aldehyde is very similar to the turnover number of the enzyme (i.e., 47 s$^{-1}$ and 43 s$^{-1}$ for $k_{\text{red}}$ and $k_{\text{cat}}$, respectively). In contrast, the overall turnover of the Ser101Ala enzyme with choline is primarily limited by the chemical step of flavin reduction with choline (e.g., with a $k_{\text{cat}}$ value of 6.7 s$^{-1}$ and a $k_{\text{red}}$ value of
6.8 s⁻¹ with choline as substrate at pH 10.0, see chapter 3) without contribution of the subsequent chemical step of flavin reduction involving the aldehyde intermediate. Since the substrate for the second reaction of flavin reduction in choline oxidase is the gem-diol species rather than the aldehyde, it is possible that the extra hydroxyl group of the gem-diol substrate compensates for the lack of the hydroxyl group on residue 101 in the mutant enzyme (7), thereby making the chemical step of flavin reduction significantly faster with betaine aldehyde than choline. The alternative possibility of betaine aldehyde being released from the active site of the Ser101Ala enzyme turning over with choline rather than proceeding through the second step of flavin reduction seems less likely based on the similarity of the three dimensional structures of the mutant and wild-type enzymes and the fact that the active site is completely secluded from the bulk solvent in choline oxidase (11, 14, 29)

Lack of a hydroxyl group on residue 101 in the active site of choline oxidase results in increased efficiencies in the oxidative half-reactions with either choline or betaine aldehyde as substrate. Evidence for this conclusion comes from the comparison of the steady state kinetic data, showing that the second-order rate constants for reaction of the reduced Ser101Ala enzyme with oxygen (k_{cat}/K_{oxygen}) were three- to four-fold larger than those observed with the wild-type enzyme. The microenvironment in proximity of the reactive C(4a) atom of the reduced flavin is considerably less hydrophilic in the Ser101Ala enzyme than in the wild-type enzyme, suggesting that this may be the cause of the increased reactivity with oxygen of the reduced flavin in the mutant enzyme. The importance of a hydrophobic microenvironment in proximity of the C(4a) atom of the flavin was recently demonstrated in choline oxidase with variant enzymes where Val464 was replaced with either alanine or threonine (14). In that case, the less hydrophobic character of the mutant enzymes at position 464, which is proximal to the C(4a) atom of the
flavin on the opposite face of the flavin than Ser101, resulted in fifty-fold decreases efficiencies in the $k_{\text{cat}}/K_{\text{oxygen}}$ value with choline as substrate for the mutant enzyme (14). Alternatively, the increased efficiencies in the oxidative half-reactions may be due to the absence of the hydrogen-bonding interaction between the O(4) atom of the flavin and the side chain of the residue 101. Interestingly, the Ser101Ala variant is the first case of increased efficiency in the oxidative half-reaction among many mutant variants of choline oxidase where no changes were observed with respect to the wild-type enzyme (His99Asn, Glu312Asp, His351Ala, and His466Ala) or where decreased efficiencies were observed (Val464Ala and Val464Thr) (10, 11, 13-15). To our knowledge, the choline oxidase Ser101Ala mutant represents the first instance in which the efficiency of a flavoprotein oxidase for the reaction of the reduced flavin with oxygen has been increased by at least three-fold with respect to the wild-type enzyme.

The flavin adduct between the C(4a) atom of FAD and oxygen that was previously observed in the crystal structure of the wild-type form of choline oxidase (11) was not observed in the structure of the Ser101Ala enzyme reported in this study. In the case of the wild-type enzyme many interactions were shown to stabilize the flavin-adduct within the active site of the enzyme, among which a hydrogen bond involving the O(4) atom of the flavin and the hydroxyl group of Ser101 (11, 29). Thus, it is possible that the absence of the flavin adduct in the Ser101Ala enzyme is due to the inability to form a hydrogen bond between the O(4) atom of the isoalloxazine ring and the side chain of residue 101. Alternatively, the lack of a flavin adduct in the structure of the Ser101Ala enzyme may simply be due to the FAD not being reduced during the data collection at the synchrotron, thereby preventing the formation of the C(4a) adduct between the reduced enzyme-bound flavin and oxygen. A more planar flavin with respect to the wild-type enzyme was recently reported for another mutant form of choline oxidase, where
Val464 was replaced with alanine (14). In that case, a significantly lowered apparent affinity of the reduced enzyme for oxygen, which could not be saturated at concentrations of oxygen as high as 1 mM, was proposed to explain the lack of a flavin adduct in the crystal structure of the mutant enzyme (14).

In conclusion, replacement of Ser101 with alanine in the active site of choline oxidase produced a variant enzyme with increased efficiencies in the oxidative half-reactions where the reduced flavin reacts with oxygen and decreased efficiencies in the reductive half-reactions with choline and the aldehyde intermediate of reaction. Interestingly, this mutation has disclosed the importance of a protein hydroxyl group in close proximity of the flavin C(4a) atom in the optimization of the overall turnover of choline oxidase, which requires fine tuning of four consecutive half-reactions for the conversion of an alcohol to a carboxylic acid.

2.6 Acknowledgment

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2.7 References


CHAPTER 3

Importance of a Serine Proximal to the C(4a)-N(5) Flavin Atoms for Hydride Transfer in Choline Oxidase

(This chapter has been published verbatim in Yuan, H. and Gadda, G., (2010), *Biochemistry* 50: 770-779)

3.1 Abbreviations

Ser101Ala enzyme, choline oxidase with Ser101 substituted with alanine; Ser101Thr enzyme, choline oxidase with Ser101 substituted with threonine; Ser101Val enzyme, choline oxidase with Ser101 substituted with valine; Ser101Cys enzyme, choline oxidase with Ser101 substituted with cysteine; Asn510His enzyme choline oxidase with Asn510 substituted with histidine; DMSO, dimethyl sulfoxide; KIE, kinetic isotope effect.

3.2 Abstract

Choline oxidase catalyzes the flavin-dependent, two-step oxidation of choline to glycine betaine with the formation of an aldehyde intermediate. In the first oxidation reaction the alcohol substrate is initially activated to its alkoxide via proton abstraction. Substrate oxidation occurs via hydride transfer from the alkoxide α-carbon to the N(5) atom of the enzyme-bound flavin. In the wild-type enzyme, proton and hydride transfers are mechanistically and kinetically uncoupled. In this study, we have mutagenized an active site serine proximal to the C(4a)-N(5) atoms of the flavin and investigated the reactions of proton and hydride transfers by using substrate and solvent kinetic isotope effects. Replacement of Ser101 with threonine, alanine, cysteine or valine resulted in biphasic traces in anaerobic reductions of the flavin with choline investigated in a stopped-flow spectrophotometer. Kinetic isotope effects established that the
kinetic phases correspond to the proton and hydride transfer reactions catalyzed by the enzyme. Upon removal of Ser101 there is at least a 15-fold decrease in the rate constants for proton abstraction, irrespective of whether threonine, alanine, valine or cysteine is present in the mutant enzymes. A logarithmic decrease spanning 4 orders of magnitude is seen in the rate constants for hydride transfer with increasing hydrophobicity of the side chain at position 101. This study shows that the hydrophilic character of a serine residue proximal to the C(4a)-N(5) flavin atoms is important for efficient hydride transfer.

3.3 Introduction

Choline oxidase (E.C.1.1.3.17; choline-oxygen 1-oxidoreductase) catalyzes the flavin-mediated oxidation of choline to glycine betaine (I). This reaction is of considerable interest for medical and biotechnological applications because the intracellular accumulation of glycine betaine, a compatible solute that does not interfere with cytoplasmic function at high concentration, is important for normal cell function under conditions of osmotic and temperature stress in bacteria and plants (30-33). Furthermore, glycine betaine is much more effective than other compatible solutes in the stabilization of the structure and function of macromolecules and the highly ordered state of membranes (34). In biotechnology, the codA gene encoding for choline oxidase is used to improve the tolerance of economically important crops like tomatoes, potatoes and rice towards environmental stresses due to hypersalinity of the soil or high and low temperatures (35-37). A membrane associated choline dehydrogenase that is homologous to the cytosolic oxidase is found in many pathogenic bacteria, where it was recently shown that the betA gene encoding for the dehydrogenase is likely associated with virulence (38). Thus, the study of the mechanism of choline oxidase offers an opportunity to control glycine betaine
biosynthesis in pathogenic bacteria and to provide osmotic balance with the environment in plants.

Choline oxidase from Arthrobacter globiformis has been investigated using biophysical (9, 17, 39, 40), structural (11, 29), mechanistic (7, 9, 41-46), computational (47), and site-directed mutagenic (11, 13-17, 48-52) approaches. The reaction of choline oxidation catalyzed by choline oxidase includes two reductive half-reactions where the FAD cofactor is reduced in subsequent steps by the alcohol substrate and an aldehyde intermediate of the reaction. Each reductive half-reaction is followed by an oxidative half-reaction where the reduced FAD cofactor is oxidized by molecular oxygen with formation of hydrogen peroxide (Scheme 3.1) (for a recent review, see (1)). In the wild-type enzyme, the first oxidation is triggered by the kinetically fast abstraction of the hydroxyl proton of choline by a catalytic base with pK_a of ~7.5, which has not been identified yet (13, 16, 49), resulting in the formation of a transient alkoxide (9, 42, 43, 46). The side chains of His351 and His466 contribute to the stabilization of the alkoxide species through hydrogen bonding and electrostatic interactions, respectively (13, 16). A rate-limiting transfer of a hydride ion from the α-carbon of the alkoxide species to the N(5) atom of the flavin results in the oxidation of choline and the reduction of the enzyme-bound flavin. This was established with solvent, substrate, and multiple kinetic isotope effects on the steady state kinetic parameter $k_{cat}/K_m$ with choline as a substrate for the enzyme (1, 43). The abstraction of the hydroxyl proton of the substrate is associated with an isomerization of the enzyme-substrate complex that is kinetically independent from the subsequent reaction of hydride ion transfer. This is suggested in the wild-type enzyme by the comparison of the kinetic isotope effects on the flavin reduction step under reversible and irreversible catalytic regimes (46), and in active site mutant enzymes with replacements of residues that are not directly involved in catalysis, namely Glu312 to
aspartate or Val464 to either alanine or threonine, by substrate or solvent kinetic isotope effects (11, 15).

Scheme 3.1 The Two-step Oxidation of Choline Catalyzed by Choline Oxidase.

In the X-ray structure of choline oxidase resolved to 1.86 Å, the Oγ atom of Ser101 is less than 4 Å from the N(5) atom of FAD and within hydrogen bonding distance (i.e., <3 Å) of the oxygen atom of DMSO (Figure 3.1), an additive that was used in the crystallization of the enzyme (11). This suggests that Ser101 may interact with the substrate and be actively involved in the oxidation of choline catalyzed by the enzyme. Moreover, the recent determination of the X-ray structure of the Ser101Ala enzyme to a resolution of 2.5 Å showed lack of structural changes in the active site and the overall structure of the enzyme upon replacing the side chain on residue101 (Figure 3.1) (52). This prompted us to investigate the role of Ser101 in the reductive half-reaction catalyzed by choline oxidase by using site-directed mutagenesis and rapid kinetics.

In the present study, the effects of replacing Ser101 with alanine, threonine, cysteine or valine on the reductive half-reaction catalyzed by choline oxidase have been investigated using both solvent and substrate kinetic isotope effects in a stopped-flow spectrophotometer. The mutated enzymes were slower than the wild-type enzyme in their ability to activate choline to the alkoxide species, resulting in biphasic reductions of the flavin in anaerobic stopped-flow reactions with choline. Kinetic isotope effects allowed us to assign the two kinetic phases of flavin reduction to the stepwise cleavages of the substrate OH and CH bonds. Finally, it was
shown that the rate of hydride ion transfer from the alkoxide to the flavin decreased with decreasing hydrophilicity of the side chain on residue 101.

Figure 3.1 Comparison of the active sites of wild-type and Ser101Ala choline oxidase. The residues with carbons in green represent the mutant structure, whereas the carbon atoms for the wild-type enzyme are in gray. The C(4a)-oxygen adduct observed with the wild-type enzyme is shown in red. The structures of wild-type and the S101A mutant enzymes are from pdb files 2JBV (11) and 3NNE (52), respectively.

3.4 Experimental Procedures

Materials. Escherichia coli strain Rosetta(DE3)pLysS was from Novagen (Madison, WI). QIAPrep Spin Miniprep kit was from Qiagen (Valencia, CA). The QuickChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Oligonucleotides for site-directed mutagenesis and sequencing of the mutant genes were from Sigma Genosys (The Woodlands, TX). 1,2-[2H4]-Choline and sodium deuterium oxide (99%) were from Isotec Inc. (Miamisburg, OH). Polyethylene glycol 6000 was from Fluka (St. Louis, MO). Choline chloride was from ICN
Pharmaceutical Inc. (Irvine, CA). All other reagents used were of the highest purity commercially available.

**Instruments.** UV-visible absorbance spectra were recorded using an Agilent Technologies model HP 8453 diode array spectrophotometer equipped with a thermostated water bath. The enzymatic activity of choline oxidase was measured polarographically using a computer-interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument Ltd.). Stopped-flow experiments were carried out using a Hi-Tech SF-61 Double Mixing Stopped Flow spectrophotometer.

**Site-directed Mutagenesis and Protein Purification.** The mutated genes for the choline oxidase variants containing alanine, threonine, cysteine or valine instead of Ser101 were prepared as previously described (39). The resulting mutant genes were sequenced at the DNA Core Facility of Georgia State University using an Applied Biosystems Big Dye Kit on an Applied Biosystems model ABI 377 DNA Sequencer to confirm the presence of the mutation. The mutant enzymes were expressed and purified to homogeneity as previously described for wild-type choline oxidase (17, 39, 42). The enzymes were stored at -20 °C in 20 mM Tris-Cl, pH 8.0, and were found to be stable for at least six months.

**Kinetic Assays.** The apparent steady state kinetic parameters at atmospheric oxygen of the mutant enzymes were measured with the method of the initial rates by monitoring oxygen consumption as described for wild-type choline oxidase (43).

Observed rate constants for flavin reduction of the enzymes were determined anaerobically at varying concentrations of substrate (choline or 1,2-[2H4]-choline) in 50 mM sodium pyrophosphate, pH 10.0, using a stopped-flow spectrophotometer thermostated at 25 °C, as previously described (11). Equal volumes of the enzyme and substrate were mixed anaerobically
in the stopped-flow spectrophotometer in the presence of a mixture of glucose/glucose oxidase (5 mM/0.5 μM) yielding a final enzyme concentration of ~10 μM. Substrate concentrations were ≥50 μM, ensuring pseudo-first order conditions. Data were collected in the dual beam mode using photomultiplier detection set at 452 nm.

For the determination of solvent kinetic isotope effects on the observed first-order rate constants for flavin reduction, all of the reagents were prepared using 99.9% deuterium oxide by adjusting the pD value with NaOD. The pD values were determined by adding 0.4 to the pH electrode readings (53). Solvent viscosity effects were measured in the presence of 0.0211g/mL PEG-6000 as viscosigen (which is equivalent to relative viscosity of 1.26) (54), in both the tonometer containing the enzyme and the syringes containing the organic substrates.

**Data Analysis.** Kinetic data were fit with KaleidaGraph software (Synergy Software, Reading, PA) and the Kinetic Studio Software Suite (Hi-TgK Scientific, Bradford on Avon, U. K.). The apparent steady state kinetic parameters at atmospheric oxygen were determined by fitting the data to the Michaelis-Menten equation for one substrate.

Stopped-flow traces were fit to eq. 1, which describes a double-exponential process; \( k_{\text{obs1}} \) and \( k_{\text{obs2}} \) represent the observed first-order rate constants associated with the absorbance changes of the fast and slow phases, \( t \) is time, \( A \) is the absorbance at 452 nm at any given time, \( B \) and \( C \) are the amplitudes of the absorbance changes for the fast and slow phases, and \( D \) is the absorbance at infinite time. The kinetic parameters associated with the fast and slow phases of flavin reduction seen in the reductive half-reaction where determined by using eqs 2 and 3, respectively (see Appendix for derivation). Here, \( k_{\text{obs1}} \) and \( k_{\text{obs2}} \) represent the observed first-order rate constants associated with the absorbance changes of the fast and slow phases at any given concentration of substrate (\( S \)); \( k_{\text{lim1}} \) and \( k_{\text{lim2}} \) are the limiting rate constants at saturated substrate concentration of
the fast and slow phases, respectively; $^{\text{app}}K_{\text{fast}}$ and $^{\text{app}}K_{\text{slow}}$ are the apparent dissociation constants defining equilibria between free enzyme and free substrate and enzyme-substrate complexes; and $c$ is a constant.

$$A = B \exp(-k_{\text{obs1}}t) + C \exp(-k_{\text{obs2}}t) + D$$ (1)

$$k_{\text{obs1}} = \frac{k_{\text{lim1}}S + c}{^{\text{app}}K_{\text{fast}} + S}$$ (2)

$$k_{\text{obs2}} = \frac{k_{\text{lim2}}S}{^{\text{app}}K_{\text{slow}} + S}$$ (3)

3.5 Results

Expression and Purification of Ser101 Mutant Enzymes. The choline oxidase variants in which Ser101 is replaced with alanine, threonine, cysteine or valine were expressed and purified by using the same protocol previously used for the wild-type enzyme (39). A mixture of air-stable anionic semiquinone and oxidized flavin species was observed throughout the purification procedure of all the mutant enzymes, as previously reported for the wild-type enzyme (9, 39). The fully oxidized and active mutant enzymes were obtained by dialysis at pH 6.0, following the procedure described for the wild-type enzyme (39). In all cases, the flavin was covalently linked to the protein as established by heat denaturation of the mutant enzymes followed by spectroscopic analysis of the samples after removal of the denatured proteins by centrifugation. The spectral properties of the oxidized Ser101 mutant enzymes are summarized in Table 3.1, along with the apparent steady state kinetic parameters determined with choline at atmospheric oxygen concentration and pH 7.0. Both the $^{\text{app}}(k_{\text{cat}}/K_m)$ and $^{\text{app}}k_{\text{cat}}$ values of the mutant enzymes were between 1.5- and 1,000-fold lower than in the wild-type enzyme, indicating that Ser101 is an important residue for catalysis in choline oxidase.
Table 3.1 Comparison of Spectral and Catalytic Properties of Ser101 Mutant Enzymes with Wild-type Choline Oxidase.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon$ (mM$^{-1}$cm$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type$^a$</td>
<td>367, 455</td>
<td>10, 11.4</td>
<td>25000</td>
<td>15</td>
<td>0.6</td>
</tr>
<tr>
<td>Ser101Thr</td>
<td>371, 450</td>
<td>8.7, 9.3</td>
<td>$18000 \pm 2000$</td>
<td>$3.0 \pm 0.1$</td>
<td>$0.17 \pm 0.02$</td>
</tr>
<tr>
<td>Ser101Ala</td>
<td>373, 452</td>
<td>8.9, 9.5</td>
<td>$4600 \pm 400$</td>
<td>$3.3 \pm 0.1$</td>
<td>$0.73 \pm 0.07$</td>
</tr>
<tr>
<td>Ser101Cys</td>
<td>374, 450</td>
<td>9.2, 9.9</td>
<td>$1100 \pm 70$</td>
<td>$0.076 \pm 0.001$</td>
<td>$0.07 \pm 0.01$</td>
</tr>
<tr>
<td>Ser101Val</td>
<td>371, 452</td>
<td>9.2, 10.9</td>
<td>$21 \pm 1$</td>
<td>$0.017 \pm 0.001$</td>
<td>$0.82 \pm 0.03$</td>
</tr>
</tbody>
</table>

$^a$ From ref. (11). $^b$ Absorbance spectra were recorded at pH 8.0, 25 °C. Enzymatic activities were measured in 50 mM potassium phosphate, pH 7.0 and 25 °C, using fully oxidized enzymes.

**Reductive Half-Reaction.** The reductive half-reactions of the Ser101Ala, Ser101Thr, Ser101Cys and Ser101Val enzymes were studied in a stopped-flow spectrophotometer by mixing anaerobic solutions of the enzyme with different concentrations of anaerobic choline at pH 10.0 and 25 °C under pseudo-first-order conditions (i.e., 0.01 mM enzyme and 0.05 mM to 5 mM choline). Alkaline pH was chosen because previous results established that catalysis is pH independent in the wild-type and a number of active site mutant enzymes of choline oxidase at pH 10.0 (7, 9, 11, 13, 16, 43, 44). The reduction of enzyme-bound oxidized FAD was monitored by the changes in absorbance at 452 nm. With all enzymes two kinetic phases were observed (Figure 3.2A). Full reduction of the enzyme-bound flavin to the anionic hydroquinone species was observed at the end of the slow kinetic phase with all the mutant enzymes and at all substrate concentrations tested, as illustrated in the example of Figure 3.2B for the Ser101Ala enzyme. In agreement with a biphasic process for the decrease in absorbance at 452 nm the stopped-flow traces with all of the mutant enzymes were fit best to eq. 1, which describes a double exponential process. When the observed first-order rate constants for the fast ($k_{\text{obs1}}$) and slow ($k_{\text{obs2}}$) phases were plotted as a function of choline concentrations they both showed saturation kinetics (Figure...
3.2C shows the example of the Ser101Ala enzyme). Accordingly, the associated kinetic parameters for the reductive half-reactions of the mutant enzymes were determined by using eqs 2 and 3 (see Appendix for derivations). With all of the mutant enzymes the fits of the rapid kinetic data yielded $\text{app}K_{\text{fast}}$ and $\text{app}K_{\text{slow}}$ values which were below the lowest concentration of choline that could be used in order to maintain pseudo first-order conditions (data not shown), thereby preventing accurate determinations of these kinetic parameters. In contrast, accurate determinations of the limiting rate constants at saturated substrate concentration for the two phases seen in the stopped-flow traces ($k_{\text{lim1}}$ and $k_{\text{lim2}}$) could be attained. As summarized in Table 3.2, the $k_{\text{lim1}}$ values for all of the Ser101 mutant enzymes were between 40 s$^{-1}$ and 125 s$^{-1}$. In contrast, the $k_{\text{lim2}}$ values spanned over two orders of magnitude between 0.016 s$^{-1}$ and 6.6 s$^{-1}$, with Thr101≈Ala101 > Cys101 > Val101 (Table 3.2). For comparison, the reductive half-reaction with the wild-type enzyme was previously shown to be monophasic with a limiting rate constant for flavin reduction of 93 s$^{-1}$ (43).

**Substrate Deuterium KIE.** Substrate KIEs were employed to probe whether the cleavage of the CH bond of choline is associated with either the fast or the slow kinetic phase observed in the stopped-flow spectrophotometer with the Ser101 mutant enzymes. The Ser101Val enzyme was too slow for an accurate determination of the KIE and was not investigated further. As exemplified in Figure 3.3 for the Ser101Ala enzyme, substitution of choline with 1,2-[2H$_4$]-choline further slowed down the slow kinetic phase observed in the stopped-flow spectrophotometer with all of the Ser101 mutant enzymes. In contrast, there were no significant changes in the fast phases for all the mutant enzymes (Figure 3.3A shows the Ser101Ala enzyme as example). Consequently, significant decreases in the $k_{\text{lim2}}$ values were observed, whereas the $k_{\text{lim1}}$ values were minimally affected by the substitution of choline with 1,2-[2H$_4$]-choline (Table
3.2). Accordingly, small substrate KIEs ≤1.1 were associated with the $k_{\text{lim1}}$ values determined with the Ser101Thr/Ala/Cys enzymes, whereas large substrate KIEs between 5.0 and 6.5 were associated with the $k_{\text{lim2}}$ values. These results establish the slow kinetic phase of flavin reduction as being primarily due to the cleavage of the substrate CH bond.

Figure 3.2 Anaerobic reduction of the Ser101 variants with choline as a substrate in 50 mM sodium pyrophosphate at pH 10.0 and 25 °C. Panel A shows the reduction traces with saturating choline for the Ser101Ala (5 mM choline; black curve), Ser101Thr (5 mM choline; blue curve), Ser101Cys (10 mM choline; red curve) and Ser101Val (10 mM choline; green) enzymes. All traces were fit with eq. 1. Time indicated is after the end of the flow, i.e. 2.2 ms. In order to improve clarity, one experimental point every 5 is shown (vertical lines). Panel B shows UV-visible absorbance spectra of the oxidized enzyme (blue) and reduced (black) species of Ser101Ala enzyme variant obtained by mixing anaerobically the oxidized enzyme with buffer and 5 mM choline in 50 mM sodium pyrophosphate buffer at pH 10.0 and 25 °C. Panel C shows the observed rate constants for the fast and slow kinetic phases as a function of choline concentration for Ser101Ala enzyme variant. Data were fit to eq. 2 and eq. 3 for the fast and slow phases, respectively.
Table 3.2 Limiting Rate Constants for the Fast and Slow Kinetic Phases of Flavin Reduction with Choline and 1,2-[2H₄]-Choline as Substrate for Ser101 Mutant Enzymes.a.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>k₃</th>
<th>k₅</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>definition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>choline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser101Thr</td>
<td>41 ± 1</td>
<td>3.07 ± 0.05</td>
</tr>
<tr>
<td>Ser101Ala</td>
<td>125 ± 2</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>Ser101Cys</td>
<td>47 ± 1</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Ser101Val</td>
<td>49 ± 1</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>Wild-type</td>
<td>no b</td>
<td>93 ± 1</td>
</tr>
<tr>
<td>1,2-[2H₄]-choline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser101Thr</td>
<td>36 ± 1</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Ser101Ala</td>
<td>125 ± 6</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>Ser101Cys</td>
<td>44 ± 1</td>
<td>0.020 ± 0.001</td>
</tr>
<tr>
<td>Wild-type</td>
<td>no b</td>
<td>10.3 ± 0.3</td>
</tr>
</tbody>
</table>

a Determined in 50 mM sodium pyrophosphate, pH 10.0 and 25°C. b Not observed

**Solvent KIE.** Solvent KIE were used with the Ser101Ala enzyme to probe whether the cleavage of the OH bond of choline is associated with the fast phase observed in the stopped-flow spectrophotometer. When water was substituted with D₂O there was a significant decrease in the observed rate constants for the fast kinetic phase at 452 nm, with minimal effects on the slow phase (Figure 3.4). The k₃ and k₅ values determined in D₂O for the Ser101Ala enzyme were 32.8 ± 0.5 s⁻¹ and 4.0 ± 0.1 s⁻¹, respectively, yielding solvent KIEs of 3.8 ± 0.1 and 1.65 ± 0.05 for the fast and slow phases observed in the stopped-flow spectrophotometer. The effect of increased solvent viscosity on the reductive half-reaction was investigated as a control for the solvent KIE, since D₂O has a relative viscosity of 1.25 at 25 °C. PEG-6000, at a concentration of 0.0211 g/mL that is equivalent to a 100% solution of D₂O, was used in the stopped-flow
spectrophotometer. The limiting rate constants at saturating concentrations of choline for both the fast and slow phases were similar to those determined in aqueous solution, yielding ratios of \((k_{\text{lim}})_{\text{H}_2\text{O}}/(k_{\text{lim}})_{\text{PEG}}\) and \((k_{\text{lim}2})_{\text{H}_2\text{O}}/(k_{\text{lim}2})_{\text{PEG}}\) values of 1.05 ± 0.01 and 0.98 ± 0.02. Lack of solvent viscosity effects establishes the solvent KIEs on the \(k_{\text{lim}}\) and \(k_{\text{lim}2}\) values as being associated with transition states with exchangeable protons being in flight rather than solvent-sensitive equilibria of enzyme-substrate complexes. These data, in turn, are consistent with the fast phase observed in the stopped-flow spectrophotometer as being due to the cleavage of the substrate OH bond.

![Graph](image)

**Figure 3.3** Anaerobic substrate reduction of the Ser101Ala enzyme with choline (black) and 1,2-[\(^{1}H_4\)]-choline (red) as substrates. The experiments were carried out in 50 mM sodium pyrophosphate, pH 10.0, at 25 °C. Panel A shows stopped-flow traces at saturating substrate concentrations. All traces were fit with eq. 1. Time indicated is after the end of the flow, i.e. 2.2 ms. In order to improve clarity, one experimental point every 5 is shown (vertical lines). Panel B shows the observed rate constants for the fast and slow kinetic phases as a function of substrate concentration. Data were fit to eq. 2 and eq. 3 for the fast phase and slow phases, respectively.
Anaerobic substrate reduction of Ser101Ala with choline as substrates in H2O (black) and D2O (red). The experiments were carried out in 50 mM sodium pyrophosphate, pH 10, at 25 °C. Panel A shows stopped-flow traces at saturating substrate concentrations. The curves were fit to eq. 1. Time indicated is after the end of the flow, i.e. 2.2 ms. In order to improve clarity, one experimental point every 5 is shown (vertical lines). Panel B shows the observed rate constants for the fast and slow kinetic phases as a function of substrate concentration. Data were fit to eq. 2 and eq. 3 for the fast and slow phases, respectively.

**Kinetic Data Simulation.** The two kinetic phases observed in the reductive half-reactions of the Ser101 mutant enzymes are consistent with the minimal kinetic model of eq 4, with the oxidized enzyme (a) giving rise to a transient species (b) that eventually decays to the reduced enzyme (c). In eq 4, a and b are reversibly connected to account for the saturation kinetics as a function of substrate concentration that is observed for the slow kinetic phase in the stopped-flow spectrophotometer (55). Figure 3.5A shows the progress curves for the relative amounts of the three species a, b and c that have been generated for the Ser101Ala enzyme upon using the experimental data with choline of Table 3.2. Similar time-courses that show the transient accumulation of the intermediate species b could be generated for the other Ser101 mutant enzymes (data not shown). Simulation of the kinetic data acquired with 1,2-[^2H_4]-choline in water clearly shows a larger accumulation of the transient species b with respect to the case with choline, as expected due to the similar rates of formation and a slower rate of decay of b when choline is substituted with 1,2-[^2H_4]-choline (cfr. black and blue curves in Figure 3.5B). Similar
accumulation of the transient species $b$, but at a delayed time, is evident in the simulation of the kinetic data with 1,2-[\textsuperscript{2}H\textsubscript{4}]choline in D\textsubscript{2}O with respect to those with choline in water, as expected due to the slowing down of the cleavage of both OH and CH bonds of the substrate (cfr. green and black curves in Figure 3.5B). Finally, a lower accumulation of the transient species $b$ is seen in the simulation of the kinetic data with choline in D\textsubscript{2}O with respect to water, as expected due to a slower rate of formation and similar rates of decay of $b$ (cfr. red and black curves in Figure 3.5B).

$$a \leftrightarrow b \Rightarrow c$$

(4)

![Figure 3.5](image.png)

Figure 3.5 Simulated reaction traces with a two-step model of $a \rightarrow b \rightarrow c$ with the equations

$$A = A_0 \exp(-k_{\text{lim}1} t)$$

$$B = \frac{A_0 k_{\text{lim}1}}{k_{\text{lim}2} - k_{\text{lim}1}} \left( \exp(-k_{\text{lim}1} t) - \exp(-k_{\text{lim}2} t) \right)$$

and

$$C = A_0 \left( 1 + \frac{k_{\text{lim}2} \exp(-k_{\text{lim}1} t) - k_{\text{lim}1} \exp(-k_{\text{lim}2} t)}{k_{\text{lim}1} - k_{\text{lim}2}} \right).$$

Kinetic parameters are taken from the experimentally determined values of Table 3.2. Panel A shows the progress curves for the three enzyme-substrate species $a$ (oxidized enzyme-substrate complex), $b$ (transient species) and $c$ (reduced enzyme-product complex) of the Ser101Ala enzyme during anaerobic substrate reduction with 10 mM choline at pH 10.0 and 25 °C. Panel B shows the time courses of species $b$ during reduction in 50 mM sodium pyrophosphate, pH 10.0, with choline in H\textsubscript{2}O (black), D\textsubscript{2}O (red), 1,2-[\textsuperscript{2}H\textsubscript{4}]choline in H\textsubscript{2}O (blue) and D\textsubscript{2}O (green) under anaerobic conditions at 25 °C.
3.6 Discussion

The reductive half-reactions of the active site variants of choline oxidase containing alanine, threonine, cysteine or valine instead of serine at position 101 were investigated to establish the role of Ser101 in the reaction of choline oxidation catalyzed by the enzyme. In the case of the Ser101Ala enzyme, the three dimensional structure of the mutant enzyme was recently shown to be practically identical to that of the wild-type enzyme, with an average rmsd value of 0.41 Å for 527 equivalent Cα atoms when the structure of the mutant enzyme is overlaid to that of the wild-type enzyme (Figure 3.1) (11, 52). In the present study we have shown that all of the mutant enzymes shared a number of kinetic, mechanistic and biochemical properties with the wild-type enzyme: 1) the UV-visible absorbance spectrum had maxima at 367-374 nm and 450-455 nm in the oxidized state and at 350-360 nm in the hydroquinone state; 2) the flavin was covalently linked to the protein; 3) an anionic flavosemiquinone was aerobically stabilized at pH 8.0 during purification; and 4) the substrate KIEs on the limiting rate constant for anaerobic flavin reduction were ≥5.0. This allowed us to compare and contrast the mechanistic properties of the enzymes and to draw conclusions on the role that is played by Ser101 in the activation of the alcohol substrate and the subsequent transfer of a hydride ion from the activated alkoxide to the flavin.

Timing of OH and CH Bond Cleavages. The abstraction of the hydroxyl proton of the substrate precedes and is mechanistically independent of the transfer of the hydride ion from the activated substrate to the flavin in the Ser101 mutant enzymes (Scheme 3.2). Evidence for this conclusion comes from the anaerobic reductive half-reactions with choline of the four variants of choline oxidase substituted on residue 101 and the associated solvent and substrate KIEs. Two kinetic phases, which are well separated from one another, were observed in the stopped-flow traces (Figure 3.2). A large solvent KIE with a value of ~4 was determined on the klim1 value for
the Ser101Ala enzyme, consistent with the fast kinetic phase reflecting the cleavage of the substrate OH bond. Negligible substrate KIEs of $\leq 1.1$ on the $k_{\text{lim}1}$ values and large substrate KIEs of $\geq 5.0$ on the $k_{\text{lim}2}$ values were seen with the Ser101Ala, Ser101Thr, and Ser101Cys enzymes, consistent with the slow kinetic phase reflecting the cleavage of the substrate CH bond.

The analytical equations that define the limiting rate constants at saturating concentration of choline for the two kinetic phases observed in the stopped-flow traces are given by eqs 5 and 6, with numbering based on Scheme 3.2 (see Appendix for derivation). These eqs simplify further to eqs 7 and 8 due to the cleavage of the substrate OH bond being significantly faster than both its reverse reaction (i.e., $k_3 > k_4$) and the cleavage of the CH bond (i.e., $k_3 > k_5$). The first condition is stipulated by the extrapolation to the origin of the hyperbola fitting the observed rate constants for the fast kinetic phase in the stopped-flow traces as a function of the concentration of choline (Figure 3.2C). The second condition is indirectly validated by the results of the KIEs since otherwise both $k_{\text{lim}1}$ and $k_{\text{lim}2}$ would incorporate both kinetic steps and be sensitive to both substrate and solvent KIEs, as illustrated by eqs 5 and 6. Thus, the timing for the cleavages of the OH and CH bonds in the Ser101 mutant enzymes is similar to that of the wild-type choline oxidase (43). What is significantly different between the two enzymes is how transition states and reaction intermediates are stabilized in the active site when the serine residue at position 101 is absent or present in the active site (vide infra).

\[
k_{\text{lim}1} = k_3 + k_4 + k_5
\]

(5)

\[
k_{\text{lim}2} = \frac{k_3 \cdot k_5}{k_3 + k_4 + k_5}
\]

(6)

\[
k_{\text{lim}1} = k_3
\]

(7)

\[
k_{\text{lim}2} = k_5
\]

(8)
**Hydroxyl Proton Abstraction.** The hydroxyl group of Ser101 stabilizes the transition state for the abstraction of the hydroxyl proton of choline. Evidence for this conclusion comes from the observation that the substitution of Ser101 with other amino acid residues results in at least a 15-fold decrease in the rate constant for the cleavage of the substrate OH bond. The fast kinetic phases seen in the stopped-flow traces of the Ser101 mutant enzymes, which report on the cleavage of the substrate OH bond (*vide ante*), have limiting rate constants at saturating choline ($k_{\text{lim}}$) comprised between 40 s$^{-1}$ and 125$^{-1}$. Instead, cleavage of the substrate OH bond in the wild-type enzyme has been shown to occur within the dead time of the stopped-flow spectrophotometer (43), which corresponds to 2.2 ms. If one considers that 6 half-lives are required to complete 98.5% of a pseudo-first order process of the type considered here$^2$, a lower limiting value of 1900 s$^{-1}$ can be estimated for the cleavage of the substrate OH bond in the wild-type choline oxidase. In agreement with substrate OH bond cleavage occurring in the dead time of the stopped-flow spectrophotometer, previous mechanistic investigations showed that the solvent KIE associated with the anaerobic reduction of the wild-type choline oxidase has a value of 0.99 (43). It is likely that in the wild-type enzyme the transition state for the OH bond cleavage is stabilized by a hydrogen bond involving the side chain of Ser101 and the oxygen atom of the alkoxide species, which is necessarily more electronegative than in the enzyme-substrate complex due to the development of the charged alkoxide intermediate (Scheme 3.3). In the mutant enzymes containing alanine, valine or cysteine in place of Ser101, such a transition state stabilization cannot be achieved, thereby making the abstraction of the substrate hydroxyl proton slow. In the case of the Ser101Thr enzyme, a sub-optimal geometry of the hydrogen bond

---

$^2$In the four enzymes mutated on Ser101 cleavage of the substrate OH bond is a first-order process (*Figure 3.2A*). It is therefore reasonable to assume that cleavage of the substrate OH bond by the wild-type enzyme is also a first-order process.
due to the presence of the extra methyl may be responsible for the slow rate of proton abstraction.

Scheme 3.2 Proposed Kinetic Mechanism for Reductive Half-Reaction of Ser101 Variants of Choline Oxidase. E, enzyme; FADox, oxidized flavin; CH, choline; FADred, reduced Flavin; BA, betaine aldehyde.

a) OH bond cleavage:

b) CH bond cleavage:

Scheme 3.3 Roles of Ser101 in the Cleavages of the OH (a) and CH (b) Bonds of Choline. Other residues roles are: E312, substrate binding (15); H351, hydrogen bonding O atom of the substrate (29); and H466, electrostatic stabilization of alkoxide species (34).

**Hydride Ion Transfer.** The hydroxyl group of Ser101 provides a hydrophilic microenvironment with hydrogen bonding capability that facilitates the transfer of the hydride
ion from the activated alkoxide to the flavin (Scheme 3.3). Evidence for this conclusion comes from a plot of the log ($k_5$) as a function of the hydrophobicity of residue 101, showing a linear decrease over 4 orders of magnitude of the rate constant for hydride transfer with decreasing hydrophilicity of residue 101 (Figure 3.6). Possible steric effects can be immediately ruled out as being responsible for the decrease in the $k_5$ values because no correlation was found when the log ($k_5$) or $k_5$ itself was plotted as a function of the volume of the side chain on residue 101 (data not shown). Among the various hydrophobicity scales that are available the best correlation was found by using the GES scale ($R^2 = 0.91$), where the hydrophobicity of the amino acids side chains is defined by the free energy for transfer of the amino acid from water to an organic solvent while taking into account the hydrogen bonding ability of the amino acid side chains (56). Interestingly, a significantly improved fit of the data was obtained when the Ser101Ala enzyme was excluded ($R^2 = 0.99$), with the latter enzyme displaying a $k_5$ value 8-times larger than the value expected from the fit of the data obtained with the other four enzymes (Figure S3.1 in SI). For comparison, $R^2$ values of 0.75 (with data for Ala101) and 0.87 (without data for Ala101) were obtained upon using the more popular Kyte hydrophobicity scale (Figure S3.2 and S3.3 in SI), where hydrophobicity is defined by the free energy for the transfer from water to vapor phase (57). These observations collectively suggest that besides its hydrophilic character, and irrespective of whether the redox potential of the flavin is affected by the substitution or not, the hydrogen bonding capability of the side chain on residue 101 is also important for the hydride transfer reaction catalyzed by choline oxidase. The presence of a somewhat mobile water molecule in place of the less mobile side chain on the Ser101Ala enzyme would readily explain why the hydride transfer reaction is significantly faster in the Ser101Ala.
**Alkoxide Intermediate vs. Transition State.** In the Ser101 mutant enzymes the alkoxide intermediate promotes detectable perturbations in the absorbance spectrum of the enzyme-bound flavin, as indicated by the decreased intensity at 450 nm ensuing in the fast kinetic phase of the stopped-flow traces\(^3\). A previous study showed that another variant of choline oxidase with the active site Asn510 substituted with histidine also catalyzes a stepwise oxidation of choline with cleavage of the OH and CH bonds occurring in the time frame of the stopped-flow spectrophotometer (\(51\)). However, in that case anaerobic reduction of the flavin was monophasic and the stepwise timing for the cleavages of the OH and CH bonds of choline could be established only by using substrate and solvent KIEs (\(51\)). A readily explanation for this apparent incongruence is that in the Ser101 mutant enzymes the alkoxide formed in catalysis is a stable reaction intermediate. In contrast, in the reaction of choline oxidation catalyzed by the Asn510His enzyme the alkoxide is a transition state. The former, being long-lived and stable, would be detected spectrophotometrically within the time frame of the stopped-flow spectrophotometer, whereas the latter would be undetectable with probes other than KIEs due to its lifetime in the \(10^{12}\) s.

\(^3\)The decrease in absorbance at 450 nm that is associated with the cleavage of the substrate OH bond (Figure 3.2A) is too large to be ascribed to the mere presence of the negatively charged alkoxide intermediate close to the flavin in the active site of the enzymes mutated on Ser101. Indeed, cleavage of the OH bond of choline did not result in absorbance changes of the type reported here in both the wild-type and a mutant form of choline oxidase where Asn510 is replaced with His (20, 30). A spectroscopic characterization of the enzyme-alkoxide intermediate in the reaction catalyzed by the Ser101 mutant enzymes is currently underway to elucidate the biophysical rationale for such a significant change in the absorbance of the oxidized enzyme-alkoxide intermediate complex. This will be presented in a separate study.
Implications for Hydride Transfer Reactions Catalyzed by Flavin-Dependent Enzymes.

Several flavin-dependent enzymes have in their active sites either a serine, threonine or tyrosine in a position that is similar, if not equivalent, to that of Ser101 of choline oxidase. Examples include, but are not limited to, class 2 dihydroorotate dehydrogenase (58), pyranose 2-oxidase (59), glucose oxidase (60), nitric oxide synthase (61), the old yellow enzyme (62), NADPH-cytochrome P450 oxidoreductase (63), alditol oxidase (64), UDP-galactose 4-epimerase (65), cholesterol oxidase (66), nitrate monooxygenase (67), heterotetrameric sacosine oxidase (68). In many instances crystallographic or site-directed mutagenesis studies have suggested that the active site serine, threonine or tyrosine residues are important for catalysis because they hydrogen bond to either the O(4) or N(5) atoms of the flavin (60-62, 64, 66, 68), the substrate in the enzyme-substrate complex (59, 65) or the transition state (63) for the reaction catalyzed. The only case in the literature for which multiple substitutions of an active site threonine (i.e., Thr178) equivalent to Ser101 of choline oxidase were engineered and the effects of the substitutions on the rate constant for the hydride transfer reaction were investigated in a stopped-flow spectrophotometer is class 2 dihydroorotate dehydrogenase (58). Interestingly, a plot of the

**Figure 3.6** Dependence of the rate constant for hydride ion transfer on the hydrophobicity of the amino acid residue at position 101 in choline oxidase. The GES scale for hydrophobicity is used (56).
log \( k_{\text{red}} \) as a function of the hydrophobicity of residue 178 shows that the rate constant for the hydride transfer reaction catalyzed by class 2 dihydroorotate dehydrogenase increases with increasing hydrophilicity of the residue 178 (Figure S3.4 in SI), as for the case presented here for choline oxidase. With dihydroorotate dehydrogenase, however, the slope of the line that fits the data to a GES hydrophobicity scale is shallower than that with choline oxidase, e.g., -0.36 ± 0.05 as compared to -1.9 ± 0.3. This suggests that the conclusions that are drawn in our study of choline oxidase, most likely in combination with other features such as the ability of the hydroxyl side chain to hydrogen bond to the 7,8-dimethyl-isoalloxazine of the flavin, may have general relevance for those flavin-dependent enzymes that utilize a hydride transfer mechanism for the oxidation of their organic substrate.

**Conclusions.** In summary, the results presented in this study on the choline oxidase variants in which serine at position 101 was replaced by alanine, cysteine, threonine or valine allow to support the conclusions that Ser101 is important for both the activation of the alcohol substrate and the subsequent hydride transfer reaction catalyzed by the enzyme. This is likely exerted by the side chain of residue 101 acting as a hydrogen bond donor to the oxygen atom of the alcohol substrate and of the alkoxide intermediate, respectively. This study represents the first instance in which the hydrophilic character of the serine residue proximal to the C(4a)-N(5) flavin atoms in a flavin-dependent enzyme, besides its hydrogen bonding capability, has been shown to be important for efficient hydride transfer from the substrate to the enzyme-bound flavin. Analysis of published data on class 2 dihydroorotate dehydrogenase indicates that the hydrophilic character of Thr178 is also important for the hydride transfer reaction catalyzed by that enzyme. In the future it would be interesting to complement these experimental findings with computational approaches to provide a physical rationale for these conclusions.
3.7 Appendix

The proposed mechanism for the reaction catalyzed by the choline oxidase Ser101 variant is described. The derivation of the equation that describes the rate of the OH bond and CH bond cleavage of the substrate follows the logic described by Bernasconi (69) and the method by Chaiyen (70).

**Scheme A3.1**

\[
\begin{align*}
&\text{CH} + \text{E-FAD}_{\text{ox}} \xrightarrow{k_1} \text{E-FAD}_{\text{ox}}-\text{CH} \xrightarrow{k_2} \text{E-FAD}_{\text{ox}}-\text{CH}_{\text{intermediate}} \xrightarrow{k_3} \text{E-FAD}_{\text{red}}-\text{BA} \\
&\text{substrate binding} \quad \text{hydroxyl proton abstraction} \quad \text{hydride transfer}
\end{align*}
\]

In **Scheme A3.1**, E-FAD_{ox} represents oxidized choline oxidase. CH is the substrate choline. BA represents bataine aldehyde. E-FAD_{red} is reduced choline oxidase. \( k_1, k_2, k_3, k_4, k_5 \) and \( k_6 \) are the rate constants. Binding step is in rapid equilibrium, therefore, \( k_{\text{obs0}} \) is very large and is in the dead time of the system and we cannot measure.

\[
\frac{d\Delta C_{\text{intermediate}}}{dt} = k_1 \cdot \Delta C_{E-\text{FADox}-\text{CH}} - (k_4 + k_5)\Delta C_{\text{intermediate}} + k_6 \cdot C_{E-\text{FADred}-\text{BA}} \cdot \Delta C_{E-\text{FADred}-\text{BA}} \quad (1)
\]

Since \( \Delta C_{E-\text{FADox}-\text{CH}} = \frac{k_1 \cdot C_{CH} \cdot \Delta C_{CH}}{k_2} \)

\[
\Delta C_{CH} + \Delta C_{E-\text{FADox}-\text{CH}} + \Delta C_{\text{intermediate}} + \Delta C_{E-\text{FADred}-\text{BA}} = 0 \quad (3)
\]

Therefore \( \Delta C_{E-\text{FADox}-\text{CH}} = \frac{k_1 \cdot C_{CH} \cdot (-\Delta C_{E-\text{FADox}-\text{CH}} - \Delta C_{\text{intermediate}} - \Delta C_{E-\text{FADred}-\text{BA}})}{k_2} \)

\[
\Delta C_{E-\text{FADox}-\text{CH}} = \frac{k_1 \cdot C_{CH} \cdot (\Delta C_{\text{intermediate}} + \Delta C_{E-\text{FADred}-\text{BA}})}{k_2 + k_1 \cdot C_{CH}}
\]

Replace the \( \Delta C_{E-\text{FADox}-\text{CH}} \) into eq. 1

\[
\frac{d\Delta C_{\text{intermediate}}}{dt} = -\frac{k_1 \cdot k_3 \cdot C_{CH}}{k_2 + k_1 \cdot C_{CH}} + k_4 + k_5)\Delta C_{\text{intermediate}} - \frac{k_1 \cdot k_3 \cdot C_{CH}}{k_2 + k_1 \cdot C_{CH}} \cdot k_6 \cdot C_{E-\text{FADred}-\text{BA}} \cdot \Delta C_{E-\text{FADred}-\text{BA}}
\]
\[
\frac{d\Delta C_{E-FAD-BA}}{dt} = k_5 \cdot \Delta C_{\text{intmediate}} - k_6 \cdot C_{E-FADred-BA} \cdot \Delta C_{E-FADred-BA}
\]

Based on the logic described by Bernasconi (69), let \( a_{11} = \frac{k_1 \cdot k_3 \cdot C_{CH}}{k_2 + k_1 C_{CH}} + k_4 + k_5 \),

\[
a_{12} = \frac{k_3 \cdot C_{CH}}{k_2 + k_1 C_{CH}} - k_6 \cdot C_{E-FADred-BA}, \quad a_{21} = k_5, \quad a_{22} = k_6 \cdot C_{E-FADred-BA}, \quad x_1 = \Delta C_{\text{intmediate}},
\]

\[
x_2 = \Delta C_{E-FADred-BA}
\]

\[
\begin{vmatrix}
a_{11} - k_{obs} & a_{12} \\
a_{21} & a_{22} - k_{obs}
\end{vmatrix}
\]

So,

\[
k_{obs1} = \frac{1}{2} (a_{11} + a_{22}) + \sqrt{\frac{1}{2} (a_{11} + a_{22})^2 + a_{12} a_{21} - a_{11} a_{22}}
\]

\[
k_{obs2} = \frac{1}{2} (a_{11} + a_{22}) - \sqrt{\frac{1}{2} (a_{11} + a_{22})^2 + a_{12} a_{21} - a_{11} a_{22}}
\]

By taking the sum and the product

\[
A = k_{obs1} + k_{obs2} = a_{11} + a_{22} = \frac{k_1 \cdot k_3 \cdot S}{k_2 + k_1 \cdot S} + k_4 + k_5 + k_6 \cdot C_{E-FADred-BA}
\]

\[
B = k_{obs1} \cdot k_{obs2} = a_{11} a_{22} - a_{12} a_{21} = \frac{k_1 \cdot k_3 \cdot S}{k_2 + k_1 \cdot S} (k_5 + k_6 \cdot C_{E-FADred-BA}) + k_4 k_6 \cdot C_{E-FADred-BA}
\]

The extrapolation to the origin of the curve fitting the kinetic data on the dependence of the \( k_{obs2} \) value as a function of the substrate concentration in Figures 3.2B, 3.3B and 3.4B establishes that the kinetic step \( k_6 \) is close to zero (71).
\[ -k_{\text{obs}2}^2 + k_{\text{obs2}} \cdot A - B = 0 \]

\[ k_{\text{obs}2} = \frac{A \pm \sqrt{A^2 - 4B}}{2} = \frac{A}{2} \pm \frac{A}{2} \sqrt{1 - \frac{4B}{A^2}}; \]

While \( A^2 \geq 4B \)

\[ \sqrt{1 - \frac{4B}{A^2}} \approx 1 - \frac{2B}{A^2} \]

Therefore, \( k_{\text{obs}2} = A - \frac{B}{A} \) or \( A + \frac{B}{A} \) and \( k_{\text{obs}1} = \frac{B}{A} \) or \( A - \frac{B}{A} \)

Since \( k_{\text{obs}1} > k_{\text{obs}2} \)

\[ k_{\text{obs}2} = \frac{B}{A} = \frac{k_1 \cdot k_3 \cdot k_5 \cdot S}{k_1 \cdot k_3 \cdot S + k_1 \cdot k_4 \cdot S + k_1 \cdot k_5 \cdot S + k_2 (k_4 + k_5)} \]

\[ = \frac{k_3 \cdot k_5}{k_3 + k_4 + k_5} \cdot \frac{S}{S + \frac{k_5 (k_4 + k_5)}{k_1 (k_3 + k_4 + k_5)}} \]

So \( k_{\text{lim2}} = \frac{k_3 \cdot k_5}{k_3 + k_4 + k_5}, \quad K_{\text{slow}} = \frac{k_2 (k_4 + k_5)}{k_1 (k_3 + k_4 + k_5)} \)

\[ k_{\text{obs}1} = \frac{B}{k_{\text{obs}2}} = \frac{k_1 \cdot k_3 \cdot k_5 \cdot S}{k_2 + k_1 S} \cdot \frac{k_1 (k_3 + k_4 + k_5) \cdot S + k_2 (k_4 + k_5)}{k_1 \cdot k_3 \cdot k_5 \cdot S} \]

\[ = \frac{k_1 (k_3 + k_4 + k_5) \cdot S + k_2 (k_4 + k_5)}{k_1 \cdot S + k_2} \]

\[ = \frac{k_1 (k_3 + k_4 + k_5) \cdot S + k_2 (k_4 + k_5)}{S + \frac{k_2}{k_4}} \]

So \( k_{\text{lim1}} = k_3 + k_4 + k_5, \quad K_{\text{fast}} = \frac{k_2}{k_4} \)
3.8 Support Information

**Figure S3.1** Dependence of the rate constant for hydride ion transfer on the hydrophobicity of the amino acid residue at position 101 in choline oxidase. Data were fit without considering the Ala101 enzyme. The GES scale for hydrophobicity is used (72).

**Figure S3.2** Dependence of the rate constant for hydride ion transfer on the hydrophobicity of the amino acid residue at position 101 in choline oxidase. The Kyte scale for hydrophobicity is used (72).

**Figure S3.3** Dependence of the rate constant for hydride ion transfer on the hydrophobicity of the amino acid residue at position 101 in choline oxidase. Data were fit without considering the Ala101 enzyme. The Kyte scale for hydrophobicity is used (72).
Figure S3.4 Dependence of the rate constant for hydride ion transfer on the hydrophobicity of the amino acid residue at position 178 in class 2 dihydroorotate dehydrogenase, where the wild-type is Thr178 (58). The GES scale for hydrophobicity is used (72).
3.9 Acknowledgment

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3.10 References


CHAPTER 4

Mechanism of Flavin C4a-Adduct Formation in a Choline Oxidase Variant

4.1 Abstract

In the active site of choline oxidase, serine 101 is proximal to the C4a and N5 flavin atom. In Ser101Cys mutant protonated Tris base induce a cysteinyl adduct at the flavin C4a position, which can convert to oxidized form reversibly while protonated Tris is removed for the active site. The activities for these two forms enzyme are not significantly different. We measured pH and isotope effects on the formation of adduct. Between pH 6.7 and 9.0, adduct formation was observed. Ser101Cys showed a nearly 7-fold slowing of adduct formation or breakage in D\textsubscript{2}O relative to H\textsubscript{2}O, indication that rate-limiting step involves one proton transfer to flavin N5 atom.

4.2 Introduction

Flavoenzymes contain either FMN or FAD at the active site as a cofactor which can transfer one or two electrons. The isoalloxazine ring of the flavin offers several possibilities for interaction with proteins. Most of them are noncovalently linked to the apoprotein. The first reported flavin covalently linked enzyme was mammalian succinate dehydrogenase (\textsuperscript{1}). Most of the covalently attachment occurs at either 8\textalpha-methyl or 6-position of the isoalloxazine (\textsuperscript{2}). Recently, a bicovalently linkage to both positions was found, like glucooligosaccharide oxidase and berberine bridge enzymes (\textsuperscript{3, 4}). There are several residues have been observed to be bound to the 8\textalpha position, such as histidine, cysteine or tyrosine. For the 6-position so far only cysteine residue was found in the linkage. Mutagenesis, spectrometry and kinetic studies in some enzymes have been shown that covalently linkage will affect redox poteintial, stability and electron transfer or protect flavin from deteterious modifications (\textsuperscript{5-9}).
There are also some enzymes found that can be covalently linked to C4a or N5 flavin and form intermediate adducts during reaction. Such as flavin C4a-thiol adduct in pig heart lipoamide dehydrogenase \((10)\), mercuric ion reductase \((11)\), LOV domains \((12, 13)\), C4a-hydroperoxy-FAD in pyranose 2-oxidase \((14)\), C4a oxygen adduct in choline oxidase \((15)\) and flavin-N5 adduct in nitroalkane oxidase \((16)\). Studies have shown that these kinds of adduct can be reversibly reverted to each other. Such as LOV domain can form FMN-cysteine adduct induced by blue light that slowly reverts to oxidized form in the dark \((13)\).

In the crystal structure of wild-type choline oxidase and Ser101Ala enzyme shows that Ser101 is proximal to the C4a and N5 flavin atoms \((17, 18)\). In this study, the generated Ser101Cys mutant shows that flavin C4a-cysteinyl adduct can be formed in a certain condition. The adduct enzyme and oxidized enzyme can convert to each other reversibly and the enzyme activity remains the same. In order to study this adduct, the mechanism of adduct formation was investigated using pH profile, solvent isotope effects, solvent viscosity effects proton inventories, inhibition and spectroscopic studies. The results shows that the formation of adduct was induced by the protonated Tris base at the active site of the enzyme. Moreover, the formation of flavin adduct is pH-dependent and when the pH lower than 6.5 or higher than 9, the flavin adduct will convert reversibly to oxidized form. \(^{2}D_{2}O(k_3)\) and \(^{2}D_{2}O(k_4)\) with a value around 7 shows that the rate-limiting step involves a proton flight.

### 4.3 Experimental Procedures

**Materials.** The Ser101 variants of choline oxidase were purified according to Yuan et al.\((19)\). *Escherichia coli* strain Rosetta (DE3)pLysS was from Novagen (Madison, WI). EDTA was from Fisher. Choline chloride and ampicillin were from ICN Pharmaceutical Inc. All other reagents were of the highest purity commercially available.
**Instruments.** UV-visible absorbance spectra were recorded using an Agilent Technologies model HP 8453 diode array spectrophotometer equipped with a thermostated water bath. The enzymatic activity of choline oxidase was measured polarographically using a computer-interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument Ltd.). Stopped-flow experiments were carried out using a Hi-Tech SF-61 Double Mixing Stopped Flow system.

**Expression and Purification of CHO mutant enzymes.** Permanent stocks of *E. coli* Rosetta (DE3) pLysS cell harboring plasmid pET/codAmg1-Ser101X or His99Asn were used to inoculate 7.5 L of Luria-Bertani broth medium containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol. Ser101X and His99Asn mutant enzymes were purified as described previously (19, 20).

**Enzyme Assays.** The apparent steady state kinetic parameters at atmospheric oxygen of the Ser101Cys enzyme were measured with the method of the initial rates by monitoring oxygen consumption as described for wild-type choline oxidase (21). Inhibition studies were performed by varying the concentrations of both choline and Tris base in air-saturated 50 mM sodium phosphate, pH 6.0, by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument) thermostated at 25 °C.

The formation of S101C flavin adduct experiments were carried out using an SF-61DX2 HI-TECH KinetAayst high performance stopped-flow spectrophotometer thermostatted at 25 °C. Rates of flavin adduct formation were determined at varying concentrations of Tris base (2.5-100 mM) in 50 mM sodium phosphate and pH 7.0. Equal volumes of the enzyme and Tris base were mixed aerobically in the stopped-flow spectrophotometer yielding a final enzyme concentration of ~10 µM. The pH dependence of the adduct formation was studied in the stopped-flow as described above in the pH range from 6.7 to 9.0.
For the determination of the solvent kinetic isotope effects on the formation of flavin adduct, all of the reagents were prepared as described above except that D$_2$O was used to dissolve both the enzyme and substrate, and the pH of the buffered solution containing the enzyme was determined by adding 0.4 to the pH electrode reading, account for the isotope effect on the ionization of sodium pyrophosphate (22). Solvent viscosity effects were measured in the presence of 8% (mass) glucose as viscosogen, in both the tonometer containing the enzyme and the syringes containing Tris base. The resulting relative viscosity at 25 °C was 1.25, which is slightly above the value of 1.23 representing a 100% solution of D$_2$O (23). For the proton inventories in solvents containing varying mole fractions of D$_2$O, the pD values were adjusted using DCl and NaOD based on the empirical relationship (eq 1) that exists between the pH-meter reading and the pD value at varying mole fractions of D$_2$O (n) (22).

\[
(A\Delta pH)_n = 0.076n^2 + 0.3314n
\]  

(1)

Spectral Studies. The pH dependence of the UV-visible absorbance spectra of choline oxidase was investigated as previously described (24) to determine the effect of pH on the spectral properties of the enzymes. The UV-visible absorbance of the flavin-bound variant enzyme was initially recorded after gel-filtration with 20 mM NaH$_2$PO$_4$ and 20mM Na$_4$P$_2$O$_7$ (buffer A) or 20 mM NaH$_2$PO$_4$, 20mM Na$_4$P$_2$O$_7$ and 20mM Tris (buffer B), pH 6.0 at 15 °C to a volume of ~2 mL and a final concentration of ~30 μM. The absorbance spectrum and pH of the enzyme solution were recorded upon each addition of 1-10 μL of 1 M potassium hydroxide and stirring until the pH was changed gradually and successively to ~12.

Data Analysis. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA) and the Hi-Kinetic Studio Software Suite (Hi-Tech Scientific, Bradford on Avon, U. K.). The kinetic data with inhibitor were fit to eq. 2, which describe competitive inhibition pattern of the
inhibitor versus choline. \( I \) is the concentration of inhibitor and \( K_{is} \) is the inhibition constant for the slope term.

\[
\frac{v}{e'} = \frac{k_{cat}A}{K_a \left( 1 + \frac{I}{K_{is}} \right) + A}
\]  

(2)

Stopped-flow traces were fit to eqs. 3 and 4, which describe single- and double-exponential processes, respectively. \( k_{obs}, k_{obs1} \) and \( k_{obs2} \) represent the observed first-order rate constant associated with the absorbance change at 455 nm, \( t \) is time, \( A_t \) is the absorbance at 455 nm at any given time, \( A, A_1, \) and \( A_2 \) are the amplitudes of the absorbance changes, and \( A_\infty \) is the absorbance at infinite time. Formation of flavin adduct parameters were determined by using eq. 5, where \( k_{obs} \) is the observed first-order rate constant for the formation of flavin adduct at any given concentration of Tris base, \( k_3 \) is the limiting first-order rate constant for flavin flavin adduct at saturating concentrations of Tris base, \( k_4 \) is the rate of the reverse step and \( \text{app} K_d \) is the dissociation constant for binding of Tris base to the enzyme.

\[
A_t = A \exp(-k_{obs}t) + A_\infty
\]  

(3)

\[
A_t = A_1 \exp(-k_{obs1}t) + A_2 \exp(-k_{obs2}t) + A_\infty
\]  

(4)

\[
k_{obs} = \frac{k_3A}{\text{app} K_d + A} + k_4
\]  

(5)

The pH dependence of \( k_3 \) was determined by fitting initial rate data to eq. 6, which describes a curve with a slope of +1 and a plateau region at high pH. The pH profile of the \( \text{app} K_d \) values was fit to eq. 7, which describes a curve with a slope of +1 and plateau regions at low pH. \( C \) is the pH-independent value of the kinetic parameter of interest. The pH dependences of the \( k_4 \) were determined by fitting initial rate data with eq. 8 where \( Y_L \) and \( Y_H \) are the limiting values at
low and high pH, respectively, and $K_a$ is the dissociation constant for the ionization of groups which are relevant to catalysis.

$$\log(k_3) = \log \frac{C}{1 + 10^{-pH}} 10^{-pK_a}$$

$$\log^{(app K_d)} = \log(C(1 + 10^{pH-pK_a}))$$

$$\log(k_4) = \log \frac{Y_L + Y_H \times 10^{-pK_a}}{1 + 10^{-pK_a}}$$

The effect of pH on the UV-visible absorbance spectrum was demonstrated in a plot of Δ absorbance versus wavelength, nm, and the presence of ionizable groups within the microenvironment of the flavin cofactor was determined in a plot of absorbance versus pH. The data of pH dependencies of the Ser101X absorbance spectra with buffer A was fitted to eq. 9, the data with buffer B was fitted to eq. 10 and the data for His99Asn was fitted to eq. 11, which describes a curve with a slope of 1 and -1 and plateau regions at low and high pH where $A$ and $B$ represent the absorbance values at the selected wavelength at low and high pH, respectively.

$$Y = \frac{(A \times 10^{-pK_{a1}} + B \times 10^{-pH})}{(10^{-pK_{a1} + 10^{-pH}})} + \frac{(B \times 10^{-pK_{a2} + A \times 10^{-pH}})}{(10^{-pK_{a2} + 10^{-pH}})}$$

$$Y = \frac{(A \times 10^{-pK_{a1}} + B \times 10^{-pH})}{(10^{-pK_{a1} + 10^{-pH}})} + \frac{(B \times 10^{-pK_{a2} + A \times 10^{-pH}})}{(10^{-pK_{a2} + 10^{-pH}})} + \frac{(B \times 10^{-pK_{a2} + A \times 10^{-pH}})}{(10^{-pK_{a2} + 10^{-pH}})}$$

$$Y = \frac{(Y_L \times 10^{-pK_{a1}} + Y_H \times 10^{-pH})}{(10^{-pK_{a1} + 10^{-pH}})}$$
4.4 Results

Spectroscopic Analysis of CHO-Ser101Cys. The UV-visible absorbance spectra of the CHO-Ser101Cys were recorded in 20 mM buffer, pH 8.0, as shown in Figure 4.1A. The Ser101Cys in 20 mM Tris displayed a significantly difference in the visible region with the wild-type enzyme, indicating subtle alterations of the FAD environment. The spectrum of this species exhibits an absorbance band at 378 nm with \( \varepsilon \) of 8.1 cm\(^{-1}\) mM\(^{-1}\). Comparison the spectrum of Ser101Cys in some buffers suggests that the requirement of Tris base for the formation of the flavin adduct. However, the spectra of Ser101Ala, Ser101Thr and Ser101Val are similar to wild-type enzyme and displayed typical flavin maxima in the near-UV and visible regions centered at 370 and 450 nm, as expected for an enzyme with bound flavin in the oxidized state, as shown in Figure 4.1B.

![Figure 4.1](image-url)

Figure 4.1 UV-visible absorbance spectra of S101X at pH 8.0. (A) Spectra of Ser101Cys at different buffer pH 8.0. Black curve represents 20 mM Tris buffer, Red curve represents 20 mM TES buffer, blue curve represents 20 mM HEPES buffer and green curve represents 20 mM potassium phosphate buffer. (B) Spectra recorded in 20 mM Tris and pH 8.0. Black curve represents CHO-wt, yellow curve represent CHO-Ser101Ala, Red curve represents CHO-Ser101Thr, blue curve represents CHO-Ser101Cys, and green curve represents CHO-Ser101Val.

Apparent Steady State Kinetic Studies. The apparent steady state kinetic parameters determined with choline at atmospheric oxygen concentration in 50 mM Tris buffer, at pH 8.0 with fully oxidized Ser101Cys and adduct Ser101Cys, respectively. Both the \( \text{app}(k_{\text{cat}}/K_m) \) and \( \text{app}k_{\text{cat}} \) values of the two species enzymes were not significantly different, with values of 560 ± 30
M⁻¹s⁻¹, 0.094 ± 0.001 s⁻¹ for fully oxidized Ser101Cys and 580 ± 40 M⁻¹s⁻¹, 0.086 ± 0.002 s⁻¹ for adduct Ser101Cys, respectively, indicating that the oxidized form and adduct form can be reversibly converted to each other without loss of enzyme activity.

**Tris Base Inhibition Studies.** The inhibition pattern of Tris versus choline was determined in 50 mM sodium phosphate, pH 6.0, using the steady state kinetics approach with atmosphere oxygen. Assuming that the positive charged choline is important for the substrate binding, protonated Tris base is expected to compete with the substrate choline. As shown in Figure 4.2, patterns with lines intersecting on the y-axis were observed at different inhibitor concentration (between 0 and 10 mM) in double reciprocal plots of the initial rates of reaction as a function of the substrate concentration. In accordance with the observed kinetic patterns, the best fits of the kinetic data is competitive inhibition. The relevant kinetic parameters determined were: \( \text{app} K_i = 18 ± 2 \text{ mM} \).

![Figure 4.2](image-url)  
*Figure 4.2* Inhibition of choline oxidase with Tris. Initial rates were measured in air-saturated 50 mM sodium phosphate at pH 6.0 and 25 °C. Tris concentrations were 0 (●), 2 (■), 5(○) and 10 mM (□).
Kinetics of Formation of the Flavin Adduct at pH 7.0 Induced by Binding of Tris. The formation of flavin adduct was investigated in a stopped-flow spectrophotometer at 25 °C. The CHO-Ser101Cys exhibit dramatic changes in its absorbance spectrum on titration with Tris, as shown in Figure 4.3A. Difference spectrums show the loss of some of the oxidized flavin at the 457 nm and the formation of a new component. The reactions exhibit first-order kinetics over a range of Tris concentrations (1-100 mM). At pH 7, the decrease in absorbance was fit best to a single exponential process, as illustrated in Figure 4.3B. The observed rate of the flavin adduct ($k_{obs}$) were dependent on the substrate concentration with a finite intercept on the y-axis, allowing the determination of limiting rate constant for the formation and breakage of flavin adduct ($k_{for}$ and $k_{rev}$) and the macroscopic equilibrium constant for Tris base binding at the active site of the enzyme $K_d$ (Figure 4.3C). The value of $k_{rev}$ is determined as 2.0 ± 0.3 s$^{-1}$ from the y-intercept of the plot. The value of $k_{for}$ is determined as 8.2 ± 0.3 s$^{-1}$ from the limiting value of $k_{obs}$. A generalized reaction scheme consistent with these data is shown in Scheme 4.1. Tris base binds with Ser101Cys. And after the formation of Ser101Cys-Tris complex, Tris base promotes the formation of a reversible flavin adduct.

![Scheme 4.1](https://example.com/scheme4.1.png)

**Scheme 4.1** Proposed Formation of Flaivn C4a Adduct Mechanism from the Kinetics of the Attack Steps

$a$ S101C$_{ox}$, oxidized CHO-S101C; S101C$_{adduct}$, S101C flavin C4a adduct; $k_5$, $k_6$ steps only present at the high pH reactions (pH not lower than 8.0).
Figure 4.3 Formation of flavin C4a adduct with Tris in 50 mM sodium phosphate, at pH 7.0 and 25 °C. Panel A shows spectral changes induced by additions of Tris base. Panel B shows the reduction traces with 1 mM (black), 2.5 mM (red), 5 mM (blue), 10 mM (purple), 25 mM (green), 50 mM (cyan) and 100 mM (brown) Tris base. All traces were fit with eq 3. Time indicated is after the end of the flow, i.e. 2.2 ms. For clarity, one experimental point every 20 is shown (vertical lines). Panel C shows the observed rate of flavin reduction as a function of Tris concentration. Data were fit to eq 5.

Effect of pH on the Formation of Adduct Induced by Tris. The pH dependence of the kinetic parameters formation of adduct induced by Tris were determined with stopped-flow spectrophotometer at 25 °C in the pH range from 6.7 to 9.0. Above pH 8.0, the reaction is distinctly biphasic, with a rapid decrease followed by a slower decrease at 457 nm (traces not shown here). The observed rate for the fast phase showed saturation kinetic while plotted as a function of Tris concentration. A plot of the observed rate for the slow phase was independent on
the Tris concentration with an average value of 0.7 s⁻¹ (Table 4.1). The rates for the second phase are independent on the Tris base concentration (data not shown here); probably due to the conformational change as proposed in the blue part of Scheme 4.1. At all pH values where flavin adduct can be formed, the observed rate constant for flavin adduct formation increased with Tris concentration. As shown in Figure 4.4A, the observed values for and \( k_{\text{for}} \) and \( k_{\text{rev}} \) were pH-dependent. Within this pH range, the rates increased with increasing pH, allowing an approximate pK\(_a\) = 6.7 ± 0.1 and 8.2 ± 0.4 to be determined for a group that must be deprotonated for \( k_{\text{for}} \) and \( k_{\text{rev}} \), respectively. The \( k_{\text{for}} \) is small at low pH, it increases with pH and reached a limiting value of 12.3 ± 0.8 s⁻¹ at pH 9.0. The \( \text{app} K_d \) are strongly pH-dependent, as shown in Figure 4.4B. The data are fit well to eq. 7 and give a pK\(_a\) value of 8.5 ± 0.1, suggesting that there is a protonated group involved in binding.

**Table 4.1** pH-Dependence of the Kinetic Parameters with Tris Base for Ser101Cys in 50 mM Buffer, at 25 °C

<table>
<thead>
<tr>
<th>pH</th>
<th>( k_{\text{for}}, \text{s}^{-1} )</th>
<th>( \text{app} K_d, \text{mM} )</th>
<th>( k_{\text{rev}}, \text{s}^{-1} )</th>
<th>( k_{\text{obs2}}, \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>protonated</td>
<td>deprotonate</td>
<td></td>
</tr>
<tr>
<td>6.7</td>
<td>5.3 ± 0.2</td>
<td>10.7 ± 2.1</td>
<td>10.5</td>
<td>0.17</td>
</tr>
<tr>
<td>7.0</td>
<td>8.2 ± 0.3</td>
<td>10.8 ± 2.2</td>
<td>10.5</td>
<td>0.33</td>
</tr>
<tr>
<td>7.5</td>
<td>8.1 ± 0.3</td>
<td>10.7 ± 2.1</td>
<td>9.7</td>
<td>0.97</td>
</tr>
<tr>
<td>8.0</td>
<td>10.2 ± 0.5</td>
<td>13.6 ± 3.8</td>
<td>10.3</td>
<td>3.27</td>
</tr>
<tr>
<td>8.5</td>
<td>10.4 ± 0.5</td>
<td>20.5 ± 5</td>
<td>10.3</td>
<td>10.25</td>
</tr>
<tr>
<td>9.0</td>
<td>12.3 ± 0.8</td>
<td>42 ± 9</td>
<td>10.1</td>
<td>31.9</td>
</tr>
</tbody>
</table>
Figure 4.4 pH-Dependence of $k_{\text{for}}$ (panel A solid circle), $k_{\text{rev}}$ (panel A empty circle) and $^{\text{app}}K_d$ (panel B) values of formation of flavin C4a adduct with Tris base. Reactions were performed in 50 mM buffer at 25 °C. Data were fit to eqs. 6, 7 and 8.

**Solvent KIEs.** The effects of deuterated solvent were determined to probe whether the kinetic steps involving solvent exchangeable protons are rate-limiting or not. The flavin adduct was investigated in H$_2$O and D$_2$O, and the relevant $^{D_2O}k_{\text{for}}$, $^{D_2O}k_{\text{rev}}$ and $^{D_2O}(^{\text{app}}K_d)$ values were determined at pH 8.8 and 25 °C with Tris base. Substitution of H$_2$O with D$_2$O, large solvent KIE values of $^{D_2O}k_{\text{for}}$ and $^{D_2O}k_{\text{rev}}$ with 6.1 ± 0.3 and 7.8 ± 0.6 were observed respectively, suggesting that the kinetic steps of solvent exchangeable protons are also at least partially rate limiting in the formation and breakage of adduct. There is a solvent KIE with $^{D_2O}(^{\text{app}}K_d)$ value of 1.6 ± 0.3 observed, probably due to pH effect because $K_d$ is not pH-independent at pH 8.8.

Solvent KIEs were also determined at pH 8.5 to avoid artifactual contribution arising from pH effect. At pH 8.5, a similar solvent KIE of 6.3 ± 0.2 and 7.3 ± 0.4 were found for $k_{\text{for}}$ and $k_{\text{rev}}$, respectively, suggesting that the observed values at pH 8.5 are in the pH-independent region. To establish whether the observed solvent KIEs originated from the chemical kinetics effect rather than being due to an increased viscosity of D$_2$O with respect to H$_2$O, the effects of solvent viscosity on the formation of flavin adduct were investigated. The experiment was carried out at pH 8.8 and 25 °C in solutions containing 8% glucose, which provides a relative solvent viscosity
equivalent to a 100% solution of D₂O. Similar \( k_{\text{for}} \) and \( k_{\text{rev}} \) values were observed in the presence and absence of viscosigen (ratios are 1.00 ± 0.03 and 1.09 ± 0.05 respectively). These data indicate that the observed solvent kinetic KIEs at pL 8.8 were not contributed by solvent viscosity.

**Proton Inventories.** To gain further understanding of the observed solvent KIEs on \( k_{\text{for}} \) and \( k_{\text{rev}} \), the proton inventory technique was used to gain insights into the number of exchangeable protons that produces the solvent KIEs. Proton inventories studies were investigated in solutions with varying mole fractions of deuterium oxide during the formation of flavin adduct. As shown in Figure 4.5, linear relationships were seen upon plotting the \( k_{\text{for}} \) and \( k_{\text{rev}} \) values as a function of the mole fraction of deuterated solvent, suggesting that the solvent KIE is due to a single proton in flight in the transition state for the reaction of flavin reduction.

![Graph](image)

**Figure 4.5** Proton inventories of \( k_{\text{for}} \) (solid circle) and \( k_{\text{rev}} \) (empty circle) values of formation of flavin C4a adduct at pL 8.8.

**Effects of pH on the UV-Visible Absorbance Spectra of Free Oxidized Variant Enzymes.**

The effect of pH on the UV-visible absorbance spectrum of the Ser101X variant enzyme in comparison with the wild-type enzyme was investigated respectively to determine whether the pKₐ values were affected by replacing the serine at position 101. The effects were monitored in
two buffer systems due to the observed flavin adduct of Ser101Cys induced by Tris base. In the buffer A system which is in the absence of Tris base, the UV-visible absorbance spectrum of the serine variants enzyme showed significant changes in absorbance around 390 nm and 500 nm with increasing pH which are similar to the wild-type enzyme around the same wavelength suggesting no differences in the flavin microenvironment of the enzymes (Figure S4.1 for wild-type, S101Ala, Ser101Cys, Ser101Thr and Ser101Val). A plot of the absorbance against pH for CHO-Ser101X (Figure S4.1, inset) showed two pK\textsubscript{a} values of ~8 and ~12, suggesting the presence of two ionizable groups. The UV-visible absorbance spectrum of the His99Asn variant enzyme showed significant changes in absorbance around 400 nm with increasing pH suggesting differences in the flavin microenvironment from the Ser101X enzymes (Figure S4.1). A plot of the absorbance against pH for CHO-His99Asn (Figure S4.1 insert) showed one pK\textsubscript{a} with a value of ~10 and consistent with the presence of one ionizable group. In the case of buffer B system which is in the presence of Tris base, the spectrums of wild-type still shows oxidized state at all the pH (Figure 4.6A). The observed spectral changes, along with the fitting of the data with eq.10, are consistent with the presence of three pK\textsubscript{a} values associated three ionizable groups (Figure 4.6B). As shown in Figure 4.6C, Ser101Cys is in oxidized state, when the pH is increased from 6.0 to 6.5. Further increasing of the pH to 9.5 yielded significantly spectral changes. Further increasing of pH above 9.5, the spectrum shows flavin goes back to the oxidized state. Three pK\textsubscript{a} values of of ~7, ~9.5 and ~12 were determined by fitting of the data with eq. 10 (Figure 4.6D), which is similar to the values obtained with wild-type under the same condition. The relevant pK\textsubscript{a} values for variants enzyme are summarized in Table 4.2.
Figure 4.6 Effect of pH on the spectral properties of CHO-WT and S101C in buffer B. UV-visible absorbance spectra were recorded at different pH for CHO-WT (A) and S101C (C) about 21 μM, at 15 °C. Only selected difference absorbance spectra for CHO-WT (B) and S101C (D) in the pH range between 6 and 12 are shown. Inset: UV-visible absorbance values as a function of pH, the curves are fits of the data to eq.10.
Table 4.2 Comparison of the $pK_a$ Values of Choline Oxidase Mutant Enzymes with Wild-Type.

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>WT$^a$</th>
<th>Ser101Ala</th>
<th>Ser101Cys</th>
<th>Ser101Thr</th>
<th>Ser101Val</th>
<th>His99Asn</th>
<th>WT$^b$</th>
<th>Ser101Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_{a1}$</td>
<td>8.2 ± 0.1</td>
<td>8.6 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>8.2 ± 0.1</td>
<td>-</td>
<td>7.9 ± 0.1</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>$pK_{a2}$</td>
<td>&gt;12</td>
<td>&gt;12</td>
<td>&gt;12</td>
<td>&gt;12</td>
<td>&gt;12</td>
<td>9.8 ± 0.1</td>
<td>&gt;12.0</td>
<td>&gt;12</td>
</tr>
<tr>
<td>$pK_{a3}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.3 ± 0.2</td>
<td>9.4 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$Spectrophotometric titration of CHO-Ser101X in the 20 mM sodium phosphate, sodium pyrophosphate buffer by KOH, 15 °C.

$^b$Spectrophotometric titration of CHO-Ser101X in the 20 mM sodium phosphate, sodium pyrophosphate and 20 mM Tris buffer by KOH, 15 °C.
4.5 Discussion

For the previous studies, the serine residue is located in proximity to the C4a-N5 atom of the flavin cofactor of choline oxidase and plays an important role in the flavin reduction reactions. In this paper, the Ser101Cys mutant was generated and shows the cysteine can form flavin adduct with C4a atom in a certain condition. The formation of flavin adduct was studied with stopped-flow spectrometry. From pH profile, inhibition and solvent kinetic isotope effects studies, the formation of flavin adduct was proposed as in Scheme 4.2. First, Tris base gets to the active site and lower the pKₐ of cysteine. Deprotonated cysteine will nucleophilically attack on the C4a of flavin isoalloxazine ring to create the cysteinyll-flavin adduct. The evidences for this mechanism are discussed in the following.

**Scheme 4.2** Proposed Formation of Flavin C4a Adduct Mechanism in 50 mM Sodium Pyrophosphate, pH 8.8

*Absorbance Spectrum of Ser101Cys Suggests the Formation of a Flavin C(4a)- Cysteinyll Adduct.* The absorbance of Ser101Cys in the Tris buffer exhibits maximum peak at 378 nm, suggesting the formation of a flavin C4a-cysteinyll adduct. Adduct formation is accompanied by changes in the visible absorption spectrum. It is identified as adduct formed between the sulfur of a cysteine residue and the C4a position of the flavin, based on the similarity of its spectral properties with covalent thiolate adducts observed with LOV, lipoamide dehydrogenase and mercuric ion reductase. The spectrum of sulfide-adduct close to a spectrum for endogenous heterotetrameric sarcosine oxidase adduct (λ_max=383 nm, ε=7.3 mM⁻¹cm⁻¹) (25) and spectra...
reported for 4a-thiolate adducts in lipoamide dehydrogenase ($\lambda_{\text{max}}=384$ nm, $\varepsilon=8.7$ mM$^{-1}$cm$^{-1}$) (10), LOV domain with the $\lambda_{\text{max}}=378$ nm, $\varepsilon=8.7$ mM$^{-1}$cm$^{-1}$ (26) and mercuric reductase ($\lambda_{\text{max}}=382$ nm, $\varepsilon=7.5$ mM$^{-1}$cm$^{-1}$) (11) where the complexes are formed by reaction of the flavin with an active site cysteine. The spectra that exhibit spectral characteristics similar to this are those measured for the formation of flavin C(4a)-cysteiny1 adduct which typically have a maximum absorbance at $\approx 380$ nm.

**Protonated Tris Is Important for the Formation of Flavin Adduct.** Data supporting this conclusion comes from the pH profile of the $\text{app}K_d$ and $k_{\text{rev}}$. From the pH profile study on the $\text{app}K_d$, the substrate affinity decreased while increasing the pH and a $pK_a$ value of $8.5 \pm 0.1$ was determined which implicates that there is a protonated group involved in substrate binding. Based on the $pK_a$ and $\text{app}K_d$ values, the concentration of different Tris base species were calculated. As summarized in Table 4.1, $\text{app}K_d$ for the protonated form is around 10 mM for all the pH, which indicated that the $pK_a$ value was assigned to Tris base; otherwise, different concentration for the protonated form will be obtained for all the pH. The inhibition studies showed that Tris base is a competitive inhibitor of the enzyme; therefore, Tris base is at the active site of the enzyme. These results clearly indicate that the protonated form of Tris will get into the active site and is the relevant species responsible for inducing the formation of flavin adduct. And also the $pK_a$ value about $8.2 \pm 0.4$ on the breakage of the adduct which is similar to the $pK_a$ value on the $\text{app}K_d$ could be Tris base. At high pH, Tris base will be deprotonated and the rate of adduct breakage increases. The requirement of protonated group for the formation of flavin C4a adduct are also seen in other enzymes with flavin C4a-cysteiny1 adduct, such as the adduct in lipoamide dehydrogenase only observes upon binding of NAD$^+$ (10) and the adduct formation in the mercuric ion reductase only occurs in the presence of NADP$^+$ (11). The possible
explanation was that the electrophilicity of the oxidized flavin increases by the closeby positive charge which promote the accumulation of the flavin adduct (I1).

**Both Flavin C(4a)-Cysteinyl Adduct Formation and Breakage Involving Proton Transfer Are Rate-Limiting Steps.** Evidence supporting this conclusion comes from the large solvent kinetic isotope effects and proton inventories on the observed reaction rate determined with stopped-flow spectrometer. The solvent KIE on the forward and reverse reactions with values of about ~7 indicates that solvent-derived protons are in flight during the formation and breakage of flavin adduct. In order to ascertain the number of protons in flight, a proton inventory was conducted. The proton inventory on $k_{\text{for}}$ and $k_{\text{rev}}$ is linear, indicating that a single proton transfer that participates in the flavin adduct formation and breakage. From the pH profile on $k_{\text{for}}$, there is a group need to be deprotonated with a $pK_a$ value of 6.7 for the formation of the Flavin C(4a)-Cysteinyl adduct. This group is proposed to be cysteine 101 at the active site. Therefore, the solvent isotope effects observed at pL 8.8 suggest that there is one proton in flight from the solvent or a solvent exchangeable site to the flavin N5 atom.

**Ser101 Does Not Change the Polarity of the Active Site of Choline Oxidase.** Evidence supporting this conclusion comes from the UV-visible absorbance pH titration in buffer A of the Ser101 variants of choline oxidase in comparison with that of the wild-type and His99Asn enzyme from pH 6 to 12. The UV-visible absorbance pH titration for choline oxidase wild-type and Ser101X variants enzyme from this study showed two $pK_a$ values of ~8 and ~11. In His99Asn mutant, the absence of the species with the $pK_a$ value of ~8 is therefore consistent with the His99 being responsible for the $pK_a$. A low $pK_a$ value of ~6 in a UV-visible absorbance pH titration between 5.5 and 8.5 has been shown in cholesterol oxidase from two different sources and assigned to the ionization of the histidine residues involved in the covalent attachment of the
flavin cofactor to the protein moiety in that enzyme (27). In wild-type choline oxidase, pK_a value of ~8 was obtained in a similar pH titration between pH 6 and 11 and was thought to be the ionization of the N(3) position of the isoalloxazine ring (24). A pH ranges used for the pH titrations for cholesterol oxidase and choline oxidase could only determine the low pK_a. The second pK_a could be assigned to the ionization of the N3 locus of the oxidized enzyme-bound FAD (24). The one pK_a (~10) observed in the asparagine variant enzyme is suitably attributable to the ionization of the N(3) position of the isoalloxazine ring. As summarized in Table 4.2, the pK_a values for wild-type enzyme and Ser101X variants are similar in buffer A, suggesting that the residue at position 101 did not change the polarity of the active site of choline oxidase. The UV-visible absorbance pH titration of wild-type and CHO-Ser101Cys variant in the buffer B suggests that a thermodynamic pK_a value of 9.4 was determined for Tris base. As shown in Table 4.2, pK_a values of 6.9 ± 0.1, 9.4 ± 0.1 and ~ 12 for S101C and pK_a values of 7.7 ± 0.1, 9.5 ± 0.2 and ~ 12 for the wild-type, both of them showed three ionizations. Comparing the pK_a values determined in buffer A, the three pK_a values could be assigned to ionization of His99, Tris base and the N3 locus of the oxidized FAD, respectively. The cysteine residue at the active site of the Ser101Cys mutant may affect the pK_a of His99 and cause the difference compared to the wild-type.

There Is a Commitment to Catalysis in the Formation of a Flavin C(4a)-Cysteiny1 Adduct.

Evidence for this conclusion comes from the different pK_a values for Tris base obtained in the pH-profile of the appK_d and pH titration flavin spectrum of Ser101Cys. The thermodynamic pK_a value of 9.4 observed is an intrinsic value. The different pK_a value with 8.5 is indicative of the presence of a forward commitment to form the flavin C4a adduct. Based on the equations 11 and 12, if k_3 << k_2, the appK_d = trueK_d, while k_3 is not significantly smaller than k_2, the pK_a value of appK_d...
will be shift. The pH profile studies show the $k_{\text{for}}$ will increase with increasing of pH. Therefore, at high pH, $k_3$ cannot be ignored, the pK$_a$ values for $^{\text{app}}K_d$ shifted to the left.

$$^{\text{app}}K_d = \frac{k_2 + k_3}{k_1} \tag{11}$$

$$^{\text{true}}K_d = \frac{k_2}{k_1} \tag{12}$$

**Proposed Model for the Formation of a Flavin C(4α)-Cysteiny1 Adduct.** Ser101Cys exhibits spectral properties significantly different from those of other enzymes. This difference is due to the fact that the flavin in the enzyme forms a reversible covalent 4α-adduct with a cysteine residue. As indicated in **Scheme 4.3**, in the presence of Tris base, the His99 and Cys101 will get deprotonated with a pK$_a$ of 6.9 and deprotonated cysteine residue nucleophilically attack to the flavin C4α atom. Further increasing pH, Tris base will get deprotonated which cause the breakage of flavin adduct. While the pH goes above 11, flavin N3 atom will get deprotonated. In the absence of Tris base, no flavin adduct was observed. The ionization of His99 and flavin N3 give two pKa, as shown in **Scheme 4.3** red color.

**Scheme 4.3** Proposed Formation of Flavin C4α Adduct Mechanism from pH Titration in the Present and Absent of Tris Base
4.6 Support Information

Figure S4.1 Effect of pH on the spectral properties of CHO-Ser101X and His99Asn in buffer A. Absorbance spectra were recorded at an enzyme concentration about 21 μM, at 15 °C. Only selected difference absorbance spectra in the pH range between 6 and 12 are shown. Inset: UV-visible absorbance values of CHO-Ser101X as a function of pH; the curves are fits of the data to eqs. 9 and 11.
4.7 References


hydroxyflavin mononucleotide catalyzed by C30A trimethylamine dehydrogenase, Bioorg Med Chem Lett 13, 4129-4132.


CHAPTER 5

Conformational Changes and Substrate Recognition in *Pseudomonas aeruginosa* D-Arginine Dehydrogenase

(This chapter has been published verbatim in Fu, G., Yuan, H., Li, C., Lu, C., Gadda, G. and Weber, I., (2010), *Biochemistry* 49: 8535-8545. The author’s contribution to this chapter involved the preparation and purification of the untagged wild-type enzyme and the determination of the steady state kinetic parameters with a number of D-amino acids as substrates.)

5.1 Abreviations

DADH, D-arginine dehydrogenase; LADH, L-arginine dehydrogenase; DAAD, D-amino acid dehydrogenase; pDAAO, porcine D-amino acid oxidase; hDAAO, human D-amino acid oxidase; LAAO, L-amino acid oxidase; PMS, phenazine methosulfate; RMSD, root-mean-square deviation.

5.2 Abstract

DADH catalyzes the flavin-dependent oxidative deamination of D-amino acids to the corresponding α-keto acids and ammonia. Here we report the first X-ray crystal structures of DADH at 1.06 Å resolution, and its complexes with iminoarginine (DADH<sub>red</sub>/iminoarginine) and iminohistidine (DADH<sub>red</sub>/iminohistidine) at 1.30 Å resolution. The DADH crystal structure comprises an unliganded conformation and a product-bound conformation, which is almost identical to the DADH<sub>red</sub>/iminohistidine crystal structure. The active site of DADH was partially occupied with iminoarginine product (30% occupancy) that interacts with Tyr53 in the minor conformation of a surface loop. This flexible loop forms an “active site lid”, similar to those seen in other enzymes, and may play an essential role in substrate recognition. The guanidinium side chain of iminoarginine forms a hydrogen bond interaction with the hydroxyl of Thr50 and an
ionic interaction with Glu87. In the structure of DADH in complex with iminohistidine, two alternate conformations were observed for iminohistidine where the imidazole groups formed hydrogen bond interactions with the side chains of His48 and Thr50 and either Glu87 or Gln336. The different interactions and very distinct binding modes observed for iminoarginine and iminohistidine are consistent with the 1000-fold difference in $k_{cat}/K_m$ values for D-arginine and D-histidine. Comparison of the kinetic data for the activity of DADH on different D-amino acids and the crystal structures in complex with iminoarginine and iminohistidine establishes that this enzyme is characterized by relatively broad substrate specificity, being able to oxidize positively charged and large hydrophobic D-amino acids bound within a flask-like cavity.

5.3 Introduction

All the standard amino acids except glycine can exist as either L- or D- optical isomers. L-amino acids are predominant in proteins synthesized by all living organisms. D-amino acids are important for bacteria as fundamental elements of the bacterial cell wall peptidoglycan layer (1). Several D-amino acids, D-leucine, D-methionine, D-tyrosine and D-tryptophan, were recently reported to regulate disassembly of bacterial biofilms (2). Also, specific D-amino acids have been discovered in many physiological processes in vertebrates, including humans (3). Dunlop et al. identified D-aspartate in the brain and other tissues of mammals and suggested it may play a role in regulating the development of these organs (4). D-serine was identified at significant levels in rat brain, at about a third of the level of L-serine (5). Moreover, D-serine in the rat brain is distributed in close association with N-methyl-D-aspartate (NMDA) and it may act as an endogenous agonist of the NMDA receptor in mammalian brains (5).

Conversion of L- and D- amino acids in living organisms is commonly catalyzed by racemases. Various aminoacid racemases have been identified in bacteria, archaea, and
eucaryotes including mammals. These racemases are classified into two groups: pyridoxal 5’-phosphate (PLP) -dependent and -independent enzymes (3). In mammals, D-serine racemases and D-aspartate racemases are the most intensively studied enzymes due to their involvement in cell aging and neural signaling (6-7). In bacteria, D-amino acids are deaminated by flavin-dependent dehydrogenases (8), allowing the bacteria to grow using D-amino acids as the sole carbon, nitrogen and energy source, in a concentration-sensitive manner, since some D-amino acid analogs have toxic effects on bacterial growth (9-10).

*Pseudomonas aeruginosa*, an opportunistic human pathogen, can grow with D-arginine as the sole source of carbon and nitrogen (11). D-to-L arginine racemization by two coupled dehydrogenases serves as prerequisite of D-arginine utilization through L-arginine catabolic pathways (12-13). One enzyme is an FAD-dependent catabolic DADH and the other is an NAD(P)H-dependent anabolic LADH. DADH catalyzes the conversion of D-arginine into 2-ketoarginine and ammonia, and LADH converts 2-ketoarginine to L-arginine. In order to understand the reaction mechanism and substrate specificity of DADH, the crystal structure of DADH was determined at the atomic resolution of 1.06 Å, while the structures of DADH crystallized in the presence of D-arginine and D-histidine were both determined at 1.30 Å resolution. Well-defined electron density for the non-covalently bound FAD and imino intermediate of the reaction allowed detailed analysis of the enzyme active site. A loop region with alternative conformations was identified in the DADH structure and is involved in substrate binding. Very distinct binding modes were observed for iminarginine and iminohistidine, in agreement with detailed kinetic analysis on substrate specificity reported previously (14) and in this paper. The structural characteristics described here provide insights into substrate recognition and the catalytic reaction mechanism of DADH.
5.4 Experimental Procedures

**Materials.** D-amino acids and L-amino acids were from Alfa Aesar and Sigma-Aldrich (St. Louis, MO). Phenazine methosulfate (PMS) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (St. Louis, MO). All of the other reagents were of the highest purity commercially available.

**Expression and Purification of DADH and SeMet-DADH.** Hexa-histidine-tagged DADH was expressed in *Escherichia coli* TOP10 and purified as described previously (13). No FAD was added during expression and purification. The selenomethionine (SeMet) DADH protein was prepared following a protocol with slight modifications as described in a previous study (15). DTT (10-20 mM) was incorporated in the buffer throughout the purification steps in order to avoid oxidation of selenium. Mass spectrometry confirmed that about 88% of the eight methionines were replaced by Se-Met.

**Crystallization and X-ray Data Collection.** Purified DADH and SeMet-DADH were concentrated to 6 mg/mL and 3 mg/mL, respectively, in 50 mM Tris at pH 7.5. Crystals were grown by the hanging drop vapor diffusion method using a well solution of 0.1M 2-(N-morpholino)-ethanesulfonic acid (MES) pH 6.5-7.0, 5% glycerol, and 6-10% (w/v) PEG6000. Crystals can grow to a size of 0.1-0.2 mm³ within two weeks. DADH was co-crystallized with D-arginine or D-histidine under similar conditions using a 1:10 molar ratio of the enzyme (~0.15mM) to substrate. The crystals were soaked in the reservoir solution with 20% glycerol as cryoprotectant for ~1 min and frozen immediately in liquid nitrogen. X-ray data were collected at 100 K on beamline 22-ID of the Southeast Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source, Argonne National Laboratory. Single-wavelength anomalous
diffraction (SAD) data for SeMet-DADH were collected at the wavelength of 0.97182 Å (high energy remote) on beamline 22-BM of SER-CAT at Argonne National Laboratory.

**Structure Determination and Model Refinement.** The X-ray data were integrated and scaled using HKL2000 (16). Program SGXPRO was used to perform phasing and automatic tracing with scaled but unmerged SeMet-DADH data (17). With this approach, 331 of the 381 residues (375 amino acids residues of the enzyme and the N-terminal hexa-histidine tag) (~87%) were successfully built. This model was then used for automated model building by ARP/WARP (18), in which 96% of the structure was fit. The structure of SeMet-DADH was then employed to solve the native DADH data set (1.06 Å) by molecular replacement using PHASER (19) in the CCP4i suite of programs (20). Notably, all the FAD atoms were distinctly visible in the electron density maps. FAD was refined with 100% occupancy. The structure of DADH was used to solve the structures of DADH co-crystallized with D-arginine or D-histidine by molecular replacement. Crystal structures were refined with SHELX97 (21). Manual adjustments and rebuilding were performed using the molecular graphics program COOT (22). The structures of DADH co-crystallized with D-arginine and D-histidine showed difference density for iminoarginine and iminohistidine, respectively, bound at the enzyme active site, as observed for D-amino acid oxidase crystallized in the presence of D-tryptophan (23). The D-amino acids are converted enzymatically to imino acids (R-C=NH), which then dissociate from the enzyme and are hydrolyzed to the final keto acids (R-C=O) in a non-enzymatic reaction. The iminoarginine was refined with 100% occupancy, while two alternate conformations were visible for the iminohistidine that refined to relative occupancies of 60% and 40%. Further analysis showed that the structure of DADH, which was crystallized without added D-amino acids, contained extra density for a low occupancy iminoarginine adjacent to a disordered loop showing two alternate
conformations. This structure was refined with two conformers corresponding to 70% occupancy of a ligand-free, open conformation DADH, and 30% occupancy of iminoarginine bound to a closed conformation of DADH. Higher peaks in the 2Fo-Fc electron density (>5σ) were observed for the two main-chain oxygen atoms of iminoarginine, while smaller density peaks (~1.3σ) were visible for other atoms. Therefore, in the ligand-free conformation, two water molecules were refined near the carboxylate oxygen atoms of iminoarginine. Solvent molecules were inserted at stereochemically reasonable positions based on the peak height of the 2Fo–Fc and Fo–Fc electron density maps, hydrogen bond interactions and interatomic distances. Anisotropic B-factors were refined for all the structures. Hydrogen atom positions were included in the last stage of refinement using all data.

Sequence similarity searches were performed using BLAST (24). Protein structures were superimposed on Cα atoms by using the secondary structure matching (SSM) module in COOT (25). Figures of the structures were generated with PYMOL (http://www.pymol.org).

**Enzyme Assay.** The determination of the apparent steady state kinetic parameters was carried out by measuring initial rates of reaction with an oxygen electrode at varying concentrations of the D-amino acid substrate and a fixed concentration of 1 mM for PMS as electron acceptor. The reaction mixture (1 mL) was first equilibrated with organic substrate and PMS at the desired concentrations before the reaction was started by the addition of DADH. Enzyme assays were conducted in 20 mM Tris-Cl, pH 8.7, 25°C. All reaction rates are expressed per molar concentration of enzyme-bound flavin.
5.5 Results

**Overall Structure of DADH.** The DADH was crystallized without the addition of FAD or D-amino acid, and the structure was solved in the orthorhombic space group P2₁2₁2₁ with one molecule per asymmetric unit using single anomalous dispersion (SAD) phasing and automated tracing. The structure was refined to an R-factor of 13.2% at 1.06 Å resolution. Structures were obtained also for DADH crystallized with substrates D-arginine or D-histidine under similar conditions. The structures were solved in the same space group P2₁2₁2₁ by using molecular replacement with the DADH structure as a template, and were both determined at the resolution of 1.30 Å and refined to R-factors of 13.4% and 12.8%, respectively. The crystallographic data and refinement statistics are summarized in **Table 5.1**. In the three structures, all 375 DADH residues and N-terminal hexa-histidine tag were defined clearly in the electron density map. Further analysis of the N-terminus of the structure revealed that the His-tag residues form several direct or water-mediated polar interactions with residues from symmetry related molecules, which stabilize the flexible terminus. Overall, the three structures are almost identical with pairwise RMSD values of 0.10-0.11 Å for 381 Cα atoms. The protein folds into two domains: an FAD-binding domain and a substrate-binding domain (**Figure 5.1**). The FAD-binding domain includes residues 1-82, 147-217 and 309-375. It consists of a central six-stranded β-sheet surrounded by five α-helices on one side and a three-stranded anti-parallel β-sheet with two α-helices on the other side. The substrate-binding domain is formed by residues 83-142 and 218-297, and consists of an eight-stranded β-sheet and two short anti-parallel β-strands forming a sandwich surrounded by four α-helices. The substrate binding site was identified near the *re* face of the FAD 7, 8-dimethyl-isoalloxazine ring at the interface of the two domains.
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| R<sub>work</sub> (%)      | 13.2       | 13.4   | 12.8      |
| R<sub>free</sub> (%)      | 15.9       | 16.6   | 16.3      |
| Mean B-factor (Å<sup>2</sup>) | 16.0   | 15.9   | 17.5      |
| Protein                   | 14.4       | 14.6   | 16.1      |
| FAD                       | 7.3        | 6.5    | 8.3       |
| Ligand                    | 29.5       | 37.0   | 16.3      |
| Water                     | 27.9       | 29.7   | 31.8      |
| Number of atoms           | 3102       | 2961   | 2971      |
| Protein                   | 53         | 53     | 53        |
| FAD                       | 12         | 12     | 22        |
| Ligand                    | 467        | 358    | 367       |
| Water                     | 0.017      | 0.013  | 0.012     |
| r.m.s. deviations         | 0.035      | 0.031  | 0.030     |

<sup>a</sup>The DADH structure contains both ligand-free and product-bound conformations.
<sup>b</sup>The SeMet DADH data were collected at the wavelength of high energy remote.
<sup>c</sup>Values in parentheses are given for the highest resolution shell.
<sup>d</sup>The angle RMSD in SHELX97 is indicated by distance in Å.
**Figure 5.1** Overall structure of DADH with iminoarginine. The DADH structure is shown in cartoon representation. Iminooarginine and cofactor FAD are shown as sticks and colored magenta and red, respectively. Loops that contribute to the active site are colored green.

**FAD-Binding Site.** DADH contains a noncovalently bound FAD cofactor as identified in the X-ray crystal structures. All non-hydrogen atoms of the FAD were clearly visible in the electron density map (**Figure 5.2A**). The flavin adopts an elongated conformation with the adenine ring buried inside the FAD-binding domain and the isoalloxazine ring located at the interface of the two domains. The adenine portion of the cofactor points toward the flavin binding domain and the 7, 8-dimethyl-isoalloxazine ring is directed towards the interface of the two domains, similar to the FADs observed in other flavin-dependent enzymes, such as L-proline dehydrogenase (26), D-amino acid oxidase (27) and L-amino acid oxidase (28-29). As illustrated in **Figure 5.2B**, the ribose ring moiety has hydrogen bond interactions with the side chain of Glu32, two water molecules and the main chain amide of Arg33. Numerous hydrogen bonds are present between the phosphate groups and the peptidyl atoms of Ala14, Thr42, Gly331 and 5 water molecules.
The central part of the conserved glycine-rich sequence GXGXXG in the N-terminal region of the polypeptide is part of an α-helix (residues 13-25) that points toward the phosphate group and is assumed to contribute to the stabilization of the two negative charges of the diphosphate(30). The 2'-OH and 4'-OH of the ribityl moiety of the cofactor establish hydrogen bond interactions with hydroxyl groups of Ser41, Thr42 and Ser45. The 3'-OH group interacts with peptidyl atoms of Gly331 and Gly334.

**Figure 5.2** (A) The 2Fo-Fc electron density map of FAD contoured at 2σ illustrates the high quality of the 1.06 Å resolution structure. FAD (colored by element type) adopts an elongated conformation. (B) Schematic diagram of the flavin-apoprotein interactions in the product-bound DADH conformation. Hydrogen bonds are indicated by dashed lines. The flavin cofactor is colored blue. For clarity, H atoms are not shown.

The 7, 8-dimethyl-isoalloxazine is sharply bent between the two planes containing the benzene and pyrimidine moieties, defining a 15° angle along the N5-N10 axis. This conformation is in agreement with several other crystallographic structures of flavin-dependent enzymes in which the flavin is in the reduced state (31). The pyrimidine portion of the 7, 8-dimethyl-isoalloxazine ring interacts through several hydrogen bonds with backbone atoms of the protein (i.e. C2=O atom with N atom of Ile335, Gln336, N3-H atom with the O atom of
His48 and a water molecule, and C=O atom with the N atom of His48). The benzene portion of the 7, 8-dimethyl-isoalloxazine ring is excluded from solvent and interacts with residues Arg44, Ser45, Ala46, Arg222, Tyr249, Gly303 and Arg305.

**Interactions of DADH with Iminoarginine.** In order to investigate the structural basis for the substrate recognition of DADH, the enzyme was crystallized with D-arginine that was converted to iminoarginine. Iminoarginine binds with the plane formed by its Cα atom, carboxylate group and imino group approximately parallel to the *re* face of the flavin, while the side chain points away from the FAD (*Figure 5.3A*). The α-carbon of the iminoarginine is 3.3 Å away from the FAD N5 atom, which is compatible with direct involvement of these atoms in the arginine oxidation catalyzed by DADH. The iminoarginine imino group is pointed out of the plane of the carboxylate atoms and can form a 2.9 Å long hydrogen bond interaction with the FAD O4 atom (*Figure 5.3B*). Several DADH residues form extensive polar interactions with the two main-chain carboxyl oxygen atoms of iminoarginine that anchor the ligand in the active site. One of the iminoarginine carboxyl oxygen forms hydrogen bonds with the side-chain hydroxyl of Tyr53, the guanidinium side chain of Arg305, and the carbonyl oxygen of Gly332. The other ligand carboxyl oxygen forms hydrogen bonds with the side-chain nitrogen of Arg222 and side-chain hydroxyl of Tyr249. Two water molecules are located near the ligand imino group, and form a hydrogen bond network extending to the imidazole side chain of His48. The side chain of iminoarginine forms hydrophobic interactions with the Val242 side chain, and a hydrogen bond interaction with the hydroxyl of Thr50. A strong ionic interaction (2.5 Å distance between pairs of O and N atoms) was observed between the guanidinium group of iminoarginine and the carboxylate group of Glu87. Hence, Glu87 may play an important role in the high specificity of
DADH for D-arginine, in agreement with the kinetic analysis on substrate specificity of DADH reported previously (14) and performed in this study (vide infra).

**Interactions of DADH with Iminohistidine.** The structure of DADH was also determined for crystals grown in the presence of D-histidine, thereby yielding a DADH<sub>red</sub>/iminohistidine complex. The iminohistidine binds to the DADH active site in two discrete conformations with clear density for the overlapping Cα atoms and carboxylate groups and weaker density for the alternate positions of the side chains (Figure 5.3C). The two iminohistidine conformations are related by a rotation of approximately 180°. In fact, there might be two additional conformations that are indistinguishable in this structure due to the potential 180° rotation of the imidazole ring. The imidazole orientation showing more hydrogen bond interactions with DADH is illustrated in Figure 5.3D. The main-chain atoms of conformation A (60% occupancy) lie nearly parallel to the isoalloxazine ring of FAD, whereas its imidazole side chain is rotated by about 30°. The iminohistidine atoms of conformation B (40% occupancy) all lie on the same plane. DADH residues Tyr53, Arg222, Tyr249, Arg305, and Gly332 form conserved polar interactions with the ligand main-chain oxygen atoms in both conformations (Figure 5.3D). The imino group of the ligand forms a polar interaction with Gly332 in conformation A and with the hydroxyl of Tyr249 in conformation B. In both conformations the side chain of iminohistidine forms hydrogen bonds with the side chain of His48 and the hydroxyl of Thr50. The imidazole group also interacts with the side chain of Glu87 and the O4 of FAD in conformation A, and the side chain of Gln336 in conformation B. When the imidazole is rotated by 180° the interactions with His48, Thr50 and Gln336 are retained for conformation B. The interactions of conformation A with Glu87 and FAD are lost, however, suggesting they are not critical since they appear in only one of four possible conformations of iminohistidine.
Figure 5.3 DADH interactions with iminoarginine (A, B) and iminohistidine (C, D). Carbons are colored yellow for DADH active site residues and green for the imino acids. FAD is represented by its isoalloxazine ring in magenta. The Fo-Fc omit map of the ligand is indicated as blue mesh and contoured at 3σ. Dashed lines represent hydrogen bonds and ionic interactions.

Conformational Flexibility of the Active Site. No substrate or other ligand was added to the protein solution during crystallization of the DADH. However, the solved ligand-free structure contained weaker density at the substrate-binding site that was fit by 30% occupancy of the
iminoarginine reaction product. It is possible that the ligand was trapped during the bacterial expression of the protein, as observed for other enzymes, for example, a tetrahedral reaction intermediate was discovered in the crystal structure of bacterial Est30 (15). The iminoarginine lies adjacent to residues 50-56 of loop L1 with two alternate conformations that were refined with 0.7/0.3 relative occupancy (Figure 5.4A). This loop had a single well-defined conformation in the electron density for structures of DADH with iminoarginine and iminohistidine that corresponds to the higher occupancy conformation in the ligand-free structure. Therefore, the crystal structure of DADH contains both ligand-free and product-bound conformations, which suggests that a conformational change occurs in the adjacent loop regions upon binding of the substrate. Comparison of the two conformations of DADH revealed distinct changes in two regions of loop L1: the residues 50-56 adjacent to the imino product and the residues 45-47 near the flavin ring of FAD.

One of the major conformational changes between the ligand-free and the product-bound conformations in the DADH structure was observed at the substrate binding site for residues 50-56 of loop L1 region (Figure 5.4B). Four loops (L1, residues 33-56; L2, 244-248; L3, 261-276; L4, 329-336) form a flask-like substrate binding pocket. The binding pocket has a small entrance but expands at the bottom near FAD. The iminoarginine product binds to DADH in a similar manner as observed in the structures of pDAAO/iminotryptophan (23) and hDAAO/iminoserine (32). In the ligand-free conformation, the side chain of Tyr53 points away from the active site and forms a hydrogen bond with Thr137, whereas in the structure of the product-bound enzyme, the aromatic ring of Tyr53 moves into the active site and forms hydrogen bonds with the carboxyl oxygen of the imino acid and also the side chains of Glu246 and Arg305. Other residues also form different interactions in the two conformations of this loop. In the ligand-free
conformation, the side chain of Thr50 forms hydrogen bonds with Asp39 and Ala52, while Gly54 and Thr55 both form polar interactions with Arg59. In the product-bound conformation, Thr50 interacts with Glu87 and Gln336, and its main-chain peptidyl oxygen forms a hydrogen bond with the main-chain amide of Tyr53. The hydroxyl side chain of Thr55 forms a hydrogen bond with Asp312. The interactions of Tyr53 and Thr50, in particular, stabilize the ligand bound in the active site, suggesting that this flexible loop L1 region may act as a lid controlling substrate accessibility to the active site. The shape and flexibility of the active site enables DADH to control the entrance of substrates and accommodate a variety of substrates.

The second region showing significant differences in the ligand-free and product-bound conformations comprised residues 45-47 at the N-terminal end of loop L1, which is located at the si face of the flavin ring (Figure 5.4C). This region possesses two conformations in the DADH structure and shows a single well-defined conformation in the structures of DADH with high occupancy products. In the ligand-free conformation, the Ser45 hydroxyl group forms a hydrogen bond with the flavin N5 atom, and the side chain of Ala46 is pointed away from FAD. In the product-bound conformation, this region is flipped about 90° with the side chain of Ser45 pointed away from FAD and the Ala46 side chain approaching closer to the FAD. This structural change causes the loss of the hydrogen bond interaction between Ser45 and FAD and, instead, a new hydrogen bond is formed between the main-chain amide of Ala46 and the N5 of FAD. A similar interaction was reported between the Ala49 of pDAAO and its FAD N5 atom (23, 27). Due to the conformational changes of residues 50-56 and 45-47 in loop L1 region, the active site of DADH shrinks by about 3.8 Å when the product is bound. The distances between the Ca atoms of Ala46 and Tyr53 are 17.5 Å and 13.7 Å in the ligand-free conformation and product-bound conformation, respectively (Figure 5.4B). Loop L2 also has a slight conformational
change and moves about 0.7 Å closer to loop L1 based on the distances between the Cα atoms of loop L1 Tyr53 and loop L2 Asp245.

Figure 5.4 Conformational flexibility of DADH structure. (A) The DADH structure comprises two conformers: a 30% occupied population with iminoarginine product, and 70% one without ligand. The iminoarginine and its adjacent loop region with low occupancy (product-bound conformation) are shown as green sticks. The Fo-Fc omit map of this region (red mesh) is contoured at 2.5σ. The corresponding loop region with high occupancy (ligand-free conformation) is shown as blue sticks. (B) Comparison of ligand-free conformation (green) and product-bound conformation (magenta) at loop L1 and L2 regions in DADH structure. Ala46, Tyr53 and iminoarginine are represented as sticks. A hydrogen bond (black dotted line) is formed between Tyr53 and iminoarginine in the product-bound conformation. (C) Conformational change of residues Ser45-Ala46-Ala47 at the si face of flavin ring. Ser45 in the ligand-free conformation (green carbon atoms) forms a 3.0 Å hydrogen bond with the FAD N5 atom (gray carbon atoms). Ala46 forms a 3.3 Å long hydrogen bond with the N5 atom of FAD in the product-bound conformation (yellow carbon atoms).
**Substrate Specificity.** The steady state kinetic parameters were determined by measuring initial rates of reaction with various D-amino acids in 20 mM Tris-Cl, pH 8.7 at 25 °C. With both D-arginine and D-histidine as substrate for DADH, the $K_m$ value for PMS is ~10 μM at pH 8.7; moreover, with both substrates the enzyme displays a ping-pong bi-bi steady state kinetic mechanism (H. Yuan and G. Gadda; unpublished results). The concentration of the electron acceptor PMS was kept fixed at 1 mM PMS, ensuring saturation of the enzyme during steady state turnover. The $k_{cat}$ values determined in this study were larger than those previously reported (14), which were expressed per molar concentration of protein rather than enzyme-bound flavin. As illustrated in Table 5.2 the enzyme showed substrate preferences for amino acids with positively charged side chains, of which D-arginine appears to be the best substrate. D-lysine, D-tyrosine and D-methionine were also good substrates. In contrast, D-glutamate, D-aspartate, L-arginine and glycine were not detectably oxidized by the enzyme. Cysteine could not be assayed due to its non-enzymatic reaction with PMS in the enzyme reaction mixture.

**DADH Recognition of Iminoarginine and Iminohistidine.** Comparison of the structures of DADH in complex with iminoarginine and iminohistidine reveals the molecular basis for the relatively broad substrate specificity of this enzyme. The internal cavity of the substrate-binding site has a triangular cross-section with a narrow entrance at the top, as shown in Figure 5.5 (DADH residues Tyr53, Gly332 and Gln336 were removed to view the internal site). The bottom of the cavity extends about 14.9 Å from loop L1 to loop L3 and 14.1 Å from loop L2 to loop L4. The depth of this substrate-binding pocket is about 10 Å from the $C\alpha$ atom of Gly54 in loop L1 to the FAD O$_2$ atom. The main chain of iminohistidine is located almost on the same plane and parallel to the isoalloxazine ring of FAD, however, the side chain of iminoarginine lies almost perpendicular to the isoalloxazine ring of FAD. The main-chain atoms of iminoarginine are very
close to those of iminohistidine, and form similar polar interactions with the DADH residues Tyr53, Arg222, Tyr249, Arg305, and Gly332. Since the binding pocket interactions with the main chain of the substrate are conserved, DADH has a broad substrate specificity for a variety of D-amino acids, as shown in Table 5.2, and reported previously (14). The different side chain interactions with DADH are presumed to be responsible for the specificity differences. The longer D-arginine side chain fits well in the large substrate-binding pocket of DADH, while the smaller D-histidine cannot fill the cavity, which is consistent with the differences in catalytic activity for the two substrates.

![Figure 5.5](image)

**Figure 5.5** Comparison of DADH structures in complex with iminoarginine and iminohistidine. DADH active site residues (green sticks) line the interior of the substrate binding pocket (Thr53, Gly332 and Gln336 were omitted for clarity). Iminoarginine (red) and iminohistidine (blue) bind to the active site in very distinct conformations. FAD is represented by its isoalloxazine ring in green.
Table 5.2 Steady State Kinetics of DADH$^a$

<table>
<thead>
<tr>
<th>substrates</th>
<th>$k_{cat}/K_m$, M$^{-1}$s$^{-1}$</th>
<th>$k_{cat}$, s$^{-1}$</th>
<th>$K_m$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-arginine</td>
<td>$(3.4 \pm 0.3) \times 10^6$</td>
<td>$204 \pm 3$</td>
<td>$0.06 \pm 0.01$</td>
</tr>
<tr>
<td>D-lysine</td>
<td>$(5.3 \pm 0.2) \times 10^5$</td>
<td>$141 \pm 3$</td>
<td>$0.26 \pm 0.01$</td>
</tr>
<tr>
<td>D-tyrosine</td>
<td>$27,600 \pm 3,800$</td>
<td>$23 \pm 1$</td>
<td>$0.8 \pm 0.1$</td>
</tr>
<tr>
<td>D-methionine</td>
<td>$14,800 \pm 600$</td>
<td>$154 \pm 3$</td>
<td>$10 \pm 1$</td>
</tr>
<tr>
<td>D-phenylalanine</td>
<td>$6,900 \pm 300$</td>
<td>$75 \pm 3$</td>
<td>$11 \pm 1$</td>
</tr>
<tr>
<td>D-histidine</td>
<td>$3,140 \pm 30$</td>
<td>$35 \pm 1$</td>
<td>$11 \pm 1$</td>
</tr>
<tr>
<td>D-leucine</td>
<td>$515 \pm 60$</td>
<td>$6.4 \pm 0.3$</td>
<td>$12 \pm 1$</td>
</tr>
<tr>
<td>D-proline</td>
<td>$420 \pm 10$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-tryptophan</td>
<td>$245 \pm 3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-isoleucine</td>
<td>$195 \pm 3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-valine</td>
<td>$47 \pm 1$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-alanine</td>
<td>$41 \pm 1$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-glutamine</td>
<td>$186 \pm 3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-asparagine</td>
<td>$16 \pm 1$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-serine</td>
<td>$3.8 \pm 0.1$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-threonine</td>
<td>$0.75 \pm 0.01$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-glutamate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-aspartate</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>L-arginine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-cysteine</td>
<td>nd$^c$</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

$^a$Enzyme activity was measured at varying concentrations of substrates and 1 mM PMS in 20 mM Tris-Cl, pH8.7, at 25 °C. $^b$Cannot be saturated with the substrate, thereby $k_{cat}$ and $K_m$ values are not reported. $^c$Not determined. PMS was reduced by cysteine.

**Structural Comparison with Related Proteins.** Determination of the crystal structure of DADH allows a detailed comparison with other mechanistically related flavin-dependent oxidoreductases. Analysis of the sequences and structures showed that DADH shares low
sequence identity (<20%) but similar three-dimensional structures with L-Proline dehydrogenase β subunit (18.5% sequence identity, RMSD 2.2 Å for 342 Ca atoms, (26)), heterotetrameric sarcosine oxidase (18.4% sequence identity, RMSD 2.2 Å for 339 Ca atoms, (33)), porcine D-amino acid oxidase pDAAO (17.2% sequence identity, RMSD 2.4 Å for 270 Ca atoms, (27)) and L-amino acid oxidase LAAO (16.4% sequence identity, RMSD 3.0 Å for 245 Ca atoms, (29)).

For a detailed comparison of the overall structure and active site geometry, we have chosen the structures of the enzyme complexes with dicarboxylate ligands: pDAAO from pig kidney in complex with iminotryptophan (PDB entry 1DDO, (23)) and LAAO from Rhodococcus opacus in complex with L-alanine (PDB entry 2JB1, (29)).

pDAAO and LAAO both are homodimeric enzymes formed by two subunits interacting through their helical domain or substrate binding domain, respectively (27-29). Structural superimposition indicates that the overall structure of DADH resembles the monomer structures of pDAAO and LAAO (Figure 5.6). DADH and LAAO share the same topology for the FAD binding domain in which a central β-sheet is surrounded by α-helixes on one side and a three-stranded anti-parallel β-sheet on the other side. However, in the FAD-binding domain of pDAAO, an α-helix is observed instead of the three-stranded anti-parallel β-sheet located in DADH (Figure 5.6A). All three enzymes contain a large β-pleated sheet in the substrate binding domain, while the orientation of this β-sheet in LAAO is significantly different from those in DADH and pDAAO. Two extra β-strands are observed for LAAO at the Gly57-Cys70 region (Figure 5.6B). Furthermore, the helical domain involved in dimerization of LAAO is absent from the structures of DADH and pDAAO.
Figure 5.6 Structural comparison of DADH with pDAAO (A) and LAAO (B). DADH is shown in green cartoon representation while pDAAO and LAAO are colored in light blue with red regions indicating their major structural differences compared to DADH. FAD is represented by sticks in green and light blue corresponding to the compared structures. The ligands are: iminoarginine in DADH (magenta), iminotryptophan in pDAAO (orange) and L-alanine in LAAO (orange).

The substrate binding sites of these three enzymes are all located near the re face of the FAD isoalloxazine ring at the interface of the FAD-binding and substrate-binding domains. Despite the opposite orientation of L-alanine bound to LAAO compared to the binding mode of ligands in the DADH and pDAAO complexes, similar protein-ligand interactions are retained among the three enzymes. The specific arrangement of active site residues is suggested to be responsible for the strict enantioselectivity of each enzyme (23, 27-29). In the pDAAO/iminotryptophan complex, the carboxylate group of the ligand forms a salt bridge with the guanidinium side chain of Arg283 (Figure 5.7A). Similar interactions are formed between L-alanine and Arg84 in the structure of LAAO/L-alanine (Figure 5.7B). However, in the DADH_red/iminoarginine complex, this ionic interaction is replaced by interactions between the ligand and two arginines: Arg222
and Arg305. The carboxylate oxygen atoms of the ligands also form polar interactions with Tyr53, Tyr249 and Gly332 of DADH and Tyr224, Tyr228 and Gly313 of pDAAO, respectively. L-alanine forms similar interactions with the polar residues Gln228 and Tyr371 of LAAO. The side chains of these ligands form distinct interactions with enzyme residues. In DADH iminoarginine forms strong ionic interactions with Glu87 and a hydrogen bond with Thr50. In contrast, in pDAAO the indole side chain of iminotryptophan is surrounded by several hydrophobic residues (Ala49, Leu51, Ile215, Ile230, and Tyr228). The short side chain of L-alanine interacts with several hydrophobic residues Trp426, Ala466 and Trp467 of LAAO. These similarities and differences in interactions are presumed to be important determinants of their different substrate specificities.

**Figure 5.7** Comparison of the active sites of DADH, pDAAO and LAAO. The active site of DADH (yellow) is superimposed on that of pDAAO (A, lightblue) and LAAO (B, lightblue) along the FAD isoalloxazine ring. Iminoarginine in DADH (magenta carbon atoms) and iminotryptophan in pDAAO (cyan carbon atoms) and L-alanine in LAAO (cyan) are shown as sticks. FAD is represented by its isoalloxazine ring.
5.6 Discussion

An “Active Site Lid” Controls Substrate Accessibility. Interestingly, the atomic resolution DADH crystal structure demonstrates two conformations corresponding to the ligand-bound and ligand-free forms. A loop covering the active site shows a substantial conformational change between the two forms. A similar loop region described as an “active site lid” was reported in the structure of pDAAO (residues 216-228, (27)). It is hypothesized that this lid switches between closed and open conformations to allow substrate binding and product release (27). A similar “loop-and-lid” structure has been assigned in some of the glucose-methanol-choline (GMC) family members, including glucose oxidase (residues 76-97, (34)), cholesterol oxidase (residues 95-109, (35)), pyranose 2-oxidase (residues 452-456, (34)), and the flavoprotein domain of cellobiose dehydrogenase (residues 289-299 (36)). Besides its role as a gate in opening and closing the enzyme active site, the active site lid may be important in determining the substrate specificity of DADH. The conformational change of the lid, especially for Tyr53, may allow DADH to accommodate bulky residues like D-phenylalanine or D-tryptophan. Similar interactions have been observed in pDAAO (Tyr224, (23)) and flavocytochrome b$_2$ (Tyr254, (37)). Furthermore, closing of the lid shields the active site and the FAD from solvent. An increase of the active site hydrophobicity caused by loop closure may facilitate the hydride transfer step leading to substrate oxidation in pDAAO (23). Similar phenomena have been described in several NAD(P)H-dependent dehydrogenases (38). Further insight into the function of this active site lid may be obtained by protein engineering studies on DADH.

Substrate Specificity. DADH is characterized by a broad substrate specificity, being able to oxidize basic and hydrophobic D-amino acids of various size (14). In the crystal structures, iminoarginine forms a strong ionic interaction with the side chain of Glu87 and a hydrogen bond
with Thr50, whereas the side chain of iminohistidine extends in a different direction and forms hydrogen bonds with His48, Thr50 and Gln336. Indeed, the steady state kinetics data show a large $k_{cat}/K_{Arg}$ value of $10^6 \text{ M}^{-1}\text{s}^{-1}$ that is 1,000-fold higher than the $k_{cat}/K_{His}$ value (Table 5.2). Kinetic data for other D-amino acids indicate that the negatively charged side chain of Glu87 is the major determinant for the specificity of DADH for the positively charged substrates D-arginine and D-lysine. Another example is seen in trypsin: residue Asp189 is responsible for its narrow selection for positively charged arginine and lysine (39-40). In addition, the hydrophobic walls (Tyr53, Met240, Val 242 and Tyr249) of the DADH active site pocket create a favorable environment for the long aliphatic and unbranched parts of the basic D-arginine and D-lysine. Furthermore, D-tyrosine, D-methionine and D-phenylalanine, which are good substrates of DADH, may form favorable van der Waals contacts with the hydrophobic walls of the active site. In contrast, DADH shows low or undetectable activities toward several D-amino acids, especially the negatively charged D-glutamate and D-aspartate.

**The Ser/Ala Switch in the FAD Binding Site.** The Ser45-Ala47 region also has two conformations corresponding to the ligand-free and product-bound conformations in the DADH structure, while this region has a well-defined single conformation in the structures of DADH_{red/iminoarginine} and DADH_{red/iminohistidine}. A hydrogen bond (O_{Ser45}—N5_{FAD}) in the ligand-free conformation is replaced by another polar interaction (N_{Ala46}—N5_{FAD}) in the product-bound conformation (Figure 5.4C). Furthermore, interactions of the FAD N5 atom with a residue structurally equivalent to Ala46 of DADH are conserved among different FAD-dependent enzymes, such as pDAAO (Ala49, (27)), yeast D-amino acids oxidase (Gly52, (41)), L-proline dehydrogenase (Gly48, (26)), flavocytochrome b2 (Ala198, (37)), and L-galactono-γ-lactone dehydrogenase (Ala113, (42)). Human DAAO shares the same sequence (V_{47}AAGL_{51})
with pDAAO near the si face of FAD ring. However, hDAAO shows a conformational shift in this region, which has been suggested to be responsible for its low binding affinity for FAD as well as the slower rate of flavin reduction (32). Furthermore, substitution of the structurally equivalent Ala113 with Gly in L-galactono-γ-lactone dehydrogenase increased the reactivity of the reduced flavin with oxygen by about 400-fold (42). Therefore, the Ser/Ala switch in DADH might be involved in structural stability and also the enzymatic properties of this protein. Further studies are required to elucidate its function.

**Structural Comparison with Related Proteins.** Structural comparison of DADH with pDAAO and LAAO revealed that the active site residues of DADH are more similar to those of pDAAO (Figure 5.7), in agreement with their specificity for D-amino acids rather than L-amino acids (14). The spatial arrangement of active residues is critically related to the enzyme enantiomeric selectivity. A mirror-symmetrical relationship of active sites has been described among enzymes with opposite stereospecificities and two different modes have been reported. One is observed between pDAAO and flavocytochrome b\textsubscript{2}, in which the active sites of the two enzymes are mirrored through the plane of isoalloxazine (on the re side in pDAAO, on the si side of flavocytochrome b\textsubscript{2}) (27). Another situation is that the substrate binding sites are mirrored through the plane perpendicular to the isoalloxazine ring, which is observed in the comparison between pDAAO and LAAO (28). The active site of DADH is highly similar to that of pDAAO, and mirror-symmetrically related to that of LAAO (Figure 5.7). This observation is in agreement with the kinetic study described previously (14) and in this paper showing that DADH can oxidize a variety of D-amino acids but not L-arginine. A similar active site arrangement has been observed in other flavoenzymes like glycolate oxidase (44), L-glutamate oxidase (45) and L-
aspartate oxidase (46), suggesting these enzymes employ a common mechanism to control enantioselectivity.

In addition to their specificity toward different enantiomers of amino acids, DADH, pDAAO and LAAO display other distinct features. For example, the ligands are held in the active site by a salt bridge formed with an arginine in pDAAO and LAAO, while two active site arginines are involved in DADH. The active sites of DADH and pDAAO are covered by a loop termed the “active site lid”. This feature was not reported in the structure of LAAO, and a funnel-shaped entrance for substrate binding was proposed instead (47). In addition to the polar interactions between the ligands and the enzyme active site residues, a hydrophobic environment has been observed in all three enzymes. In fact, all the enzymes interact with a wide range of hydrophobic amino acids (14, 23, 48). Overall, the different composition and arrangement of active site residues determine differences in substrate specificity, while critical interactions for catalysis are conserved among these mechanistically related enzymes.

**Conclusions.** Comparison of the crystal structures of DADH and its complexes with iminoarginine and iminohistidine has highlighted important structural differences that rationalize the catalytic activities and substrate specificity of the enzyme. The imino products of D-arginine and D-histidine bind to the active site in very distinct side-chain conformations. Glu87 forms strong electrostatic interactions with iminoarginine, which is likely responsible for the high selectivity of DADH for positively charged residues like D-arginine and D-lysine. A loop region has been designated as an active site lid controlling the substrate accessibility to the active site, similar to those reported in other flavin-dependent enzymes. Structural comparison of DADH with other related flavin-dependent enzymes reveals that the spatial arrangement of active site residues is essential for the differences in enzyme enantioselectivity, while some specific
interactions needed for catalysis are conserved among the enzymes. Overall, the high-resolution structures for DADH described in this study will provide new guidelines for future studies of similar flavin-dependent enzymes.

5.7 Acknowledgment

We thank Dr. Johnson Agniswamy and Dr. Yuan-Fang Wang for providing help with refinement and valuable discussions. We are especially grateful for the assistance of Dr. Zheng-Qing Fu and the staff at the SER-CAT beamline at the Advanced Photon Source, Argonne National Laboratory, for assistance during X-ray data collection. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

5.8 References


CHAPTER 6

Steady State Kinetic Mechanism and Reductive Half-Reaction of D-Arginine Dehydrogenase from *Pseudomonas aeruginosa*

(This chapter has been published verbatim in Yuan, H., Fu, G., Brooks, P., Weber, I. and Gadda, G. (2010), *Biochemistry* 49: 9542-9550. The author contributed to this chapter by carrying out all of the experiments and directing the research of Mr. Phillip Brooks in the initial purification of the untagged enzyme. The crystallographic figures were prepared by Mr. Guoxing Fu.)

6.1 Abbreviations

dNTPs, deoxynucleotide triphosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride; DEAE, Diethyl-Amino-Ethyl.

6.2 Abstract

D-Arginine dehydrogenase from *Pseudomonas aeruginosa* catalyzes the oxidation of D-arginine to iminoarginine, which is hydrolyzed in solution to ketoarginine and ammonia. In the present study, we have genetically engineered an untagged form of the enzyme that was purified to high levels and characterized in its kinetic properties. The enzyme is a true dehydrogenase that does not react with molecular oxygen. Steady state kinetics studies with D-arginine or D-histidine as substrate and PMS as the electron acceptor established a ping-pong bi-bi kinetic mechanism. With the fast substrate D-arginine a dead-end complex of the reduced enzyme and the substrate occurs at high concentrations of D-arginine yielding substrate inhibition, while the overall turnover is partially limited by the release of the iminoarginine product. With the slow substrate D-histidine the initial Michaelis complex undergoes an isomerization involving multiple conformations that are not all equally catalytically competent for the subsequent oxidation reaction, while the overall turnover is at least partially limited by flavin reduction. The
kinetic data are interpreted in view of the high-resolution crystal structures of the iminoarginine- and iminohistidine-enzyme complexes.

6.3 Introduction

D-Arginine dehydrogenase is a novel flavin-dependent enzyme with FAD as a cofactor (1). It was recently isolated from *Pseudomonas aeruginosa*, a Gram-negative soil bacterium that is well known to be an important opportunistic human pathogen. The enzyme from *P. aeruginosa* has been cloned and expressed in *Escherichia coli* (2). It catalyzes the oxidation of D-arginine to iminoarginine, which is non-enzymatically hydrolyzed to α-ketoarginine and ammonia, as illustrated in Scheme 6.1 (2-4). Coupled with an anabolic NAD(P)H-dependent L-arginine dehydrogenase, the products of the D-arginine dehydrogenase oxidation of D-arginine, α-ketoarginine and ammonia, are converted into L-arginine (1). The proposed physiological function of D-arginine dehydrogenase in *Pseudomonas* is to contribute the first stage in a two-enzyme coupled system to racemize D-arginine in the D- to L-arginine conversion (1).

\[
\begin{align*}
\text{NH}_3^+ & \quad \text{COO}^- \\
R & \quad H
\end{align*}
\]

\[
\begin{align*}
\text{FAD} & \quad FADH_2 \\
\text{unknown e' acceptor} & \quad \text{R} \quad \text{COO}^- \\
\text{R} \quad \text{NH}_2^+ & \quad \text{NH}_3^+ \\
& \quad H \\
\text{R} & \quad \text{NH}_2^+ \\
& \quad \text{N} \\
& \quad \text{NH}_2^+
\end{align*}
\]

\[
\begin{align*}
\text{R} & \quad \text{COO}^- \\
& \quad + \text{H}_2\text{O} \\
\text{R} & \quad \text{COO}^- + \text{NH}_4^+
\end{align*}
\]

**Scheme 6.1** Oxidation of D-Arginine by D-Arginine Dehydrogenase.

The biochemistry of D-amino acid catabolism has been poorly studied in comparison to that of L-amino acids. In general, D-amino acids can be metabolized after conversion into the L-enantiomers by racemase (5). Alternatively, D-amino acids can be utilized by D-amino acid oxidase, which is an FAD-dependent enzyme and plays an important role in microbial
metabolism (6). D-Amino acid oxidase was first studied by Krebs in 1935 (7) and is now well characterized. The enzyme has FAD noncovalently bound to the polypeptide and exhibits optimal activity toward neutral D-isomers of amino acids and lower efficiency toward basic ones (8). The reduced FAD is then reoxidized by molecular oxygen to yield hydrogen peroxide (8).

D-Arginine dehydrogenase is characterized by broad substrate specificity, being able to oxidize D-amino acids of various size and polarity (9). All naturally occurring D-amino acids except for D-glutamate, D-aspartate and glycine are oxidized by the enzyme, with the best substrate, D-arginine, displaying the highest $k_{cat}/K_m$ with a value of $3 \times 10^6$ M$^{-1}$s$^{-1}$ (9). D-Arginine dehydrogenase was first described in 1988 by Hass et al. (2). Recently, the dauA gene encoding for D-arginine dehydrogenase was cloned and used to express a His-tagged version of the enzyme that was characterized crystallographically at high resolutions (i.e., $\leq 1.3$ Å) in complex with either iminoarginine or iminohistidine (1, 9). The physiological electron acceptor of the enzyme has not been identified yet (1).

In the present study, we have genetically engineered an untagged form of D-arginine dehydrogenase, purified it to high levels and investigated its steady state kinetic mechanism and reductive half-reaction with the physiological substrate D-arginine and with D-histidine. The kinetic data presented are interpreted in view of the high resolution structures of D-arginine dehydrogenase reported recently (1, 9).

### 6.4 Experimental Procedures

**Materials.** The plasmid pCR3 harboring the dauA gene encoding for D-arginine dehydrogenase was a gift from Prof. Chung-Dar Lu at Georgia State University. E. coli strain Rosetta(DE3)pLysS was from Novagen (Madison, WI). Ndel, BamHI, alkaline phosphatase, T4 DNA ligase, dNTPs, BSA, lambda DNA/EcoRI + Hind III markers and MgCl$_2$ were purchased
from Promega (Madison, WI); cloned Pfu DNA polymerase was from Stratagene (La Jolla, CA); QIAprep and QIAquick purification kits were from Qiagen (Valencia, CA); Luria-Bertani agar, Luria-Bertani broth, chloramphenicol, IPTG, lysozyme, sodium hydrosulfite (dithionite), D-arginine, phenazine methosulfate (PMS) and PMSF were obtained from Sigma-Aldrich (St. Louis, MO). Ampicillin, agarose and electrophoresis-grade agar were purchased from ICN Biomedicals (Aurora, OH). Oligonucleotides were from Sigma Genosys (The Woodlands, TX). D-Histidine was from Alfa Aesar (Ward Hill, MA). All of the other reagents were of the highest purity commercially available.

**Plasmid Construction.** Plasmid pCR3 harboring the *dauA* gene encoding for D-arginine dehydrogenase (1) was employed as template to construct a recombinant plasmid for the expression of untagged enzyme using standard PCR amplification methods. Forward and reverse oligonucleotide primers were designed incorporating *Nde*I and *Bam*HI as the 5’ and 3’ cloning sites, respectively. The PCR product was then ligated into the expression vector pET20b(+) cleaved with the same restriction enzymes to ensure directional cloning to remove the N-terminal His-tag. The resulting plasmid pET/PA3863 was confirmed by sequencing and transformed into *E. coli* strain DH5α.

**Gene Expression and Enzyme Purification.** Permanent frozen stocks of *E. coli* cells Rosetta(DE3)pLysS harboring the plasmid pET/PA3863 were used to inoculate 50 mL of Luria-Bertani broth medium containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol and cultures were grown at 37 °C for 16 hours to be used as a preculture. The starting precultures (48 mL) were used to inoculate 7.5 L Luria-Bertani broth medium containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol. When the cultures reached optical densities of ~0.6 at
600 nm, the temperature was lowered to 18 °C and IPTG was added to a final concentration of 0.1 mM. After 18 hours the cells were harvested by centrifugation at 20,000 g for 20 min at 4 °C.

All purification steps were carried out at 4 °C. The wet cell paste was suspended in 0.1 mM PMSF, 0.2 mg/mL lysozyme, 1 mM EDTA, 10% glycerol and 20 mM Tris-Cl, pH 8.0, in a ratio 1 g wet cell paste to 4 mL of lysis buffer. The suspended cells were then allowed to incubate with stirring for 30 min on ice with 20 μg/mL RNase and 50 μg/mL DNase in the presence of 10 mM MgCl₂. The resulting slurry was sonicated 5 times for 5 min, with 2 min intervals, before removing the cell debris by centrifugation at 20,000 g for 20 min. Solid ammonium sulfate was slowly added to the cell free extract to achieve 30% saturation. After incubating for 30 min on ice, the insoluble fraction was removed by centrifugation and discarded. The supernatant was brought up to 65% ammonium sulfate saturation, and the pellet fraction was collected by centrifugation after 30 min of stirring. The resulting pellet was suspended in 20 mM Tris-Cl, pH 8.0, 10% glycerol, and dialyzed over a period of 18 hours with four buffer changes. After dialysis, the precipitated proteins were removed by centrifugation at 20,000 g for 20 min and the supernatant was loaded directly onto a DEAE-Sepharose Fast Flow column (3 × 28 cm), equilibrated with 20 mM Tris-Cl pH 8.0, 10% glycerol. The column was eluted with 2 volumes of the same buffer, followed by a linear gradient from 0 to 0.5 M NaCl over 1 liter. The fractions with the highest purity as judged by enzymatic activity and UV-visible absorbance spectroscopy were pooled together and concentrated with the addition of 65% ammonium sulfate saturation followed by centrifugation. After centrifugation, the resulting pellet was suspended in 20 mM Tris-Cl, pH 8.7, 10% glycerol, and dialyzed against four changes of the same buffer. After removal of the precipitated protein by centrifugation, the enzyme was stored at -20 °C.
**Enzyme Assay.** The concentration of D-arginine dehydrogenase was determined with the method of Bradford (10) by using the Bio-Rad protein assay kit with BSA as standard. The enzymatic activity of D-arginine dehydrogenase was measured by monitoring the initial rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument) at 25 °C in which PMS (1 mM) was used as the primary electron acceptor and the enzymatically reduced PMS was spontaneously reoxidized by molecular oxygen. The reaction was started with the addition of D-arginine dehydrogenase to 1 mL reaction mixture, with the final concentration of 9.9 nM enzyme and 20 mM D-arginine. One unit of enzymatic activity corresponds to the consumption of one μmol of oxygen per min.

First-order rate constants for flavin reduction were determined at varying concentrations of D-arginine (0.1-0.5 mM) or D-histidine (2.5-100 mM) in 20 mM Tris-Cl, pH 8.7, using a stopped-flow spectrophotometer thermostated at 25 °C. Equal volumes of the enzyme and D-arginine or D-histidine were mixed anaerobically in the stopped-flow spectrophotometer following established procedures (11) yielding a final enzyme concentration of ~10 μM. The stopped-flow traces were not changed when the experiment was carried out aerobically.

The determination of the steady state kinetic parameters was carried at varying concentrations of D-arginine (0.02-2 mM) or D-histidine (1-70 mM) and PMS (0.005-0.5 mM), in 20 mM Tris-Cl, pH 8.7, 25 °C. The reaction mixture (1 mL) was first equilibrated with the substrates at the desired concentrations before the reaction was started with the addition of the enzyme to a final concentration of ~ 0.01 μM. Enzyme assays were conducted in 20 mM Tris-Cl, pH 8.7. Initial rates of reaction were expressed per molar content of enzyme-bound flavin. Solvent viscosity effects on steady state kinetic parameters were measured in 20 mM Tris-Cl, pH
8.7, 25 °C using glycerol as viscosigen. The values for the relative viscosities of glycerol-containing solutions were taken from Weast (12) and adjusted for 25 °C.

**Data Analysis.** Data analysis was carried out by using KaleidaGraph software (Synergy Software, Reading, PA), Enzfitter software (Biosoft, Cambridge, UK) or the Kinetic Studio Software Suite (Hi-Tech Scientific, Bradford on Avon, U. K.). Stopped-flow traces were fit to eq. 1, which describes a single exponential process, where \( k_{\text{obs}} \) represents the observed first-order rate constant for flavin reduction at any given concentration of substrate, \( t \) is time, \( A \) is the absorbance at 446 nm at any given time, \( B \) is the amplitude of the absorbance change, and \( C \) is the absorbance at infinite time. Kinetic parameters for the reductive half-reactions were determined by using eq. 2, where \( k_{\text{obs}} \) is the observed first-order rate constant for the reduction of the enzyme-bound flavin at any given concentration of substrate (\( S \)), \( k_{\text{red}} \) is the limiting first-order rate constant for flavin reduction at saturating concentrations of substrate, and \( \text{app} K_d \) is the apparent dissociation constant for binding of the substrate to the enzyme.

\[
A = B \exp (-k_{\text{obs}} t) + C \quad (1)
\]

\[
k_{\text{obs}} = \frac{k_{\text{red}} S}{\text{app} K_d + S} \quad (2)
\]

The steady state kinetic parameters at varying concentrations of both D-arginine and PMS were determined by fitting the initial rate data to eq. 3, which describes a ping-pong mechanism with substrate inhibition (13). Data with D-histidine were fit with eq. 4, which describes a ping-pong steady state kinetic mechanism. In these equations, \( e \) represents the concentration of enzyme, \( k_{\text{cat}} \) is the turnover number of the enzyme at saturating concentrations of both the amino acid and PMS, \( K_a \) and \( K_b \) represent the Michaelis constants for the amino acid (\( A \)) and PMS (\( B \)), respectively, and \( K_{i-A} \) is the substrate inhibition constants for D-arginine.
\[
\frac{v}{e} = \frac{k_{\text{cat}}ABK_{i-A}}{K_aBK_{i-A} + K_hAK_{i-A} + ABK_{i-A} + K_hA^2}
\]

(3)

\[
\frac{v}{e} = \frac{k_{\text{cat}}AB}{K_aB + K_hA + AB}
\]

(4)

The effects of solvent viscosity on the \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{\text{Arg}}\) values were fit to eq. 5; those on the \(k_{\text{cat}}/K_{\text{His}}\) values were fit to eq. 6. In these equations \((k)_o\) and \((k)_\eta\) are the kinetic parameters of interest in the absence and presence of the viscosigen, \(S\) is the degree of viscosity dependence, and \(\eta_{\text{rel}}\) is the relative viscosity of the aqueous buffered solution, \(Y_H\) is the limiting value of the steady state kinetic parameter of interest at high concentration viscosigen.

\[
\frac{(k)_o}{(k)_\eta} = S(\eta_{\text{rel}} - 1) + 1
\]

(5)

\[
\frac{(k)_o}{(k)_\eta} = \frac{1}{1 + \frac{Y_H \times (\eta_{\text{rel}} - 1)}{(\eta_{\text{rel}} - 1) + S}}
\]

(6)

6.5 Results

Expression and Purification of Untagged Enzyme. Recombinant D-arginine dehydrogenase was expressed in E. coli strain Rosetta(DE3)pLysS and purified via 30-65% ammonium sulfate precipitation and anionic-exchange chromatography at pH 8.0 in the presence of 10% glycerol. The purity of the enzyme was confirmed by SDS-PAGE analysis. The UV-visible absorbance spectrum of the purified enzyme showed bands at 365 nm and 446 nm (data not shown), consistent with the presence of oxidized flavin bound to the enzyme. Table 6.1 summarizes the purification procedure.
Table 6.1 Purification of Untagged *P. aeruginosa* D-Arginine Dehydrogenase Expressed in *E. coli* Rosetta(DE3)pLysS

<table>
<thead>
<tr>
<th>steps</th>
<th>total protein (mg)</th>
<th>total activity (µmol O₂min⁻¹)</th>
<th>specific activity (µmol O₂min⁻¹mg⁻¹)</th>
<th>yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell-free extract</td>
<td>5,600</td>
<td>91,200</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>30-65% saturation of (NH₄)₂SO₄</td>
<td>4,700</td>
<td>100,000</td>
<td>21</td>
<td>110</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>900</td>
<td>43,000</td>
<td>48</td>
<td>47</td>
</tr>
</tbody>
</table>

Enzymatic activity was measured with 20 mM D-arginine and 1 mM PMS as substrates in air-saturated 20 mM Tris-Cl, pH 8.7 and 25 °C.

**Oxygen Reactivity.** The reactivity of D-arginine dehydrogenase with molecular oxygen was investigated by monitoring oxygen consumption over time upon mixing 0.3 µM enzyme with 0.5 mM D-arginine in a Clark-type oxygen electrode at pH 8.7 and 25 °C. As shown in Figure 6.1, in the absence of any organic electron acceptor there was no detectable consumption of oxygen. In contrast, upon addition of 1 mM PMS there was a rapid depletion of oxygen in the enzyme reaction mixture (i.e., with an estimated \( v_o/e \) value of \( \approx 800 \) s⁻¹ with 0.5 mM D-arginine). Similar results were obtained with D-lysine, D-histidine, D-serine, D-threonine, D-tyrosine, D-asparagine, D-glutamine, D-alanine, D-valine, D-leucine, D-isoleucine, D-phenylalaine, D-methionine, D-proline, and D-tryptophan as a substrate (data not shown). These data unequivocally establish the enzyme as a true dehydrogenase with very poor, if any, reactivity of the reduced flavin with molecular oxygen.
Figure 6.1 Lack of reactivity of D-arginine dehydrogenase with molecular oxygen. The assay mixture contained 20 mM Tris-Cl, pH 8.7, 0.5 mM D-arginine, at 25 °C. After monitoring the background for 1 min, 0.3 µM D-arginine dehydrogenase was added to enzyme reaction mixture and the oxygen trace was monitored. 1.5 min later, 1 mM PMS was added and the rate of the enzymatic reaction was monitored.

**Time-Resolved Flavin Reduction with D-Arginine or D-Histidine.** The reductive half-reactions in which the enzyme-bound flavin is reduced with D-arginine or D-histidine were investigated in a stopped-flow spectrophotometer at pH 8.7 and 25 °C. With D-arginine under conditions of pseudo-first order (i.e., 10 µM enzyme and ≥ 50 µM D-arginine after mixing), ~70% of the decrease in absorbance at 446 nm occurred within the dead-time of the instrument (i.e., 2.2 ms). From the final part of the reduction process that could be monitored, similar $k_{\text{obs}}$ values ~700 s⁻¹ were determined with 50, 100, or 500 µM D-arginine. These data did not allow for an accurate determination of the kinetic parameters $k_{\text{red}}$ and $K_d$, but are at least consistent with a fast process of flavin reduction ($k_{\text{red}} \geq 700$ s⁻¹) and suggest a $K_d$ value for D-arginine significantly lower than 50 µM. With D-histidine as a substrate, the decrease in absorbance at 446 nm associated with the reduction of the enzyme-bound flavin was monophasic at all the
concentrations of substrate and fit best to a single exponential process (Figure 6.2A). The observed rate constants were hyperbolically dependent on the concentration of D-histidine (Figure 6.2B), allowing for the determination of the rate constant for flavin reduction ($k_{\text{red}} = 60 \pm 1\ \text{s}^{-1}$) and the apparent equilibrium constant for substrate dissociation at the active site of the enzyme ($^{\text{app}}K_d = 10 \pm 1\ \text{mM}$). The best fit of the data was obtained with the curve extrapolating to a $y$-intercept value of zero, consistent with an irreversible reduction of the flavin.

**Figure 6.2** Anaerobic reduction of the D-arginine dehydrogenase with D-histidine as a substrate in 20 mM Tris-Cl, pH 8.7 and 25 °C. Panel A shows the reduction traces with 2.5 mM (cyan), 5 mM (blue), 10 mM (red), 20 mM (light green), 50 mM (black) and 100 mM (dark green) D-histidine. All traces were fit with eq 1. Time indicated is after the end of the flow, i.e. 2 ms. For clarity one experimental point every 10 is shown (vertical lines). Panel B shows the observed rate of flavin reduction as a function of D-histidine concentration. Data were fit to eq 2.

**Steady State Kinetic Mechanism.** The steady state kinetic mechanism and the associated kinetic parameters of the enzyme were determined using the method of the initial rates (14). The rate of oxygen consumption was measured at varying concentrations of either D-arginine or D-histidine and the artificial electron acceptor PMS, in 20 mM Tris-Cl at pH 8.7 and 25 °C. As illustrated in Figure 6.3, parallel lines were obtained in double reciprocal plots of the enzymatic rate versus the concentration of the amino acid substrate, with significant substrate inhibition at concentrations of D-arginine ≥0.5 mM. Substrate inhibition was also seen with PMS at
concentrations of D-arginine equal to, or lower than, 50 μM (data not shown). Nonetheless, the best fit of the data with D-arginine was obtained by using eq. 3, which describes a ping-pong bi-bi steady state kinetic mechanism with inhibition by D-arginine (13). The best fit of the data with D-histidine was obtained by using eq. 4, consistent with a ping-pong bi-bi steady state kinetic mechanism and no substrate inhibition. As summarized in Table 6.2, the overall rate of enzymatic turnover determined with D-arginine at saturating concentrations of both substrates \(k_{\text{cat}}\) was \(~200\) s\(^{-1}\), a value that was 6-fold larger than the \(k_{\text{cat}}\) value of 35 s\(^{-1}\) determined when D-histidine was used as substrate. The second-order rate constant for the capture of D-arginine to yield enzyme-substrate complexes committed to catalysis (i.e., \(k_{\text{cat}}/K_{\text{Arg}}\)) was in the \(10^6\) M\(^{-1}\)s\(^{-1}\) range, which was 850-fold larger than the corresponding value of 4,000 M\(^{-1}\)s\(^{-1}\) determined with D-histidine. These data establish that D-histidine is significantly slower than D-arginine as a substrate.

**Solvent Viscosity Effect.** The effects of solvent viscosity on the \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{\text{m}}\) values with D-arginine and D-histidine were investigated to determine whether diffusion-controlled events influenced the binding of the substrate and the release of the product to and from the enzyme. With D-arginine as substrate, when the reciprocals of the normalized \(k_{\text{cat}}/K_{\text{m}}\) values determined at increasing concentrations of glycerol were plotted as a function of the relative viscosity, the data yielded a line with negligible slope (i.e. 0.004 ± 0.022) (Figure 6.4A). In contrast, a line with slope of 0.14 ± 0.02 was obtained in a plot of the normalized \(k_{\text{cat}}\) value (Figure 6.4A). With D-histidine, the normalized \(k_{\text{cat}}\) values were independent of solvent viscosity; in contrast, the \(k_{\text{cat}}/K_{\text{m}}\) values increased with increasing viscosity yielding an inverse hyperbolic pattern in a plot of the \((k_{\text{cat}}/K_{\text{m}})_{\theta}/(k_{\text{cat}}/K_{\text{m}})_{0}\) values versus relative viscosity (Figure 6.4B). These data establish that
kinetic steps involving substrate binding and product release are affected in different fashions by solvent viscosity when the enzyme turns over with D-arginine or D-histidine.

**Figure 6.3** Steady state kinetics for the oxidation D-arginine (Panel A) or D-histidine (Panel B) catalyzed by D-arginine dehydrogenase. Initial rates of reaction were measured at varying concentrations of both the amino acid substrate and PMS in 20 mM Tris-Cl, pH 8.7, at 25 °C. Panel A: Concentrations of PMS were (○) 500 μM; (■) 100 μM; (□) 20 μM; (▲) 10 μM and (▲) 5 μM. Data were fit to eq. 3. Panel B: Concentrations of PMS were (■) 100 μM; (□) 50 μM; (▲) 10 μM; and (▲) 5 μM. Data were fit to eq. 4.

**Figure 6.4** Effects of solvent viscosity on the steady state kinetic parameters for the D-arginine dehydrogenase with D-arginine or D-histidine as a substrate. Panel A shows the normalized $k_{\text{cat}}$ (●) and $k_{\text{cat}}/K_{m}$ (■) values with D-arginine as a function of the relative solvent viscosity. Panel B shows the normalized $k_{\text{cat}}$ (●) and $k_{\text{cat}}/K_{m}$ (■) values with D-histidine as a function of the relative solvent viscosity. The dashed lines with a slope of 1 indicate the expected results for a fully diffusion-limited reaction. The values for the relative viscosities of the solvent were taken from Weast (12) and adjusted for 25 °C. Reaction rates were measured at varying concentrations of D-arginine or D-histidine and fixed 1 mM PMS in 20 mM Tris-Cl, pH 8.7, at 25 °C.
Table 6.2 Kinetic Parameters of D-Arginine Dehydrogenase with D-Arginine and D-Histidine $^a$

<table>
<thead>
<tr>
<th>kinetic parameters</th>
<th>D-arginine</th>
<th>D-histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$, s$^{-1}$</td>
<td>204 ± 3</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>$K_a$, mM</td>
<td>0.06 ± 0.01</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>$K_b$, µM</td>
<td>11 ± 2</td>
<td>8.0 ± 0.6</td>
</tr>
<tr>
<td>$k_{cat}/K_a$, M$^{-1}$s$^{-1}$</td>
<td>(3.4 ± 0.3) × 10$^6$</td>
<td>4,000 ± 300</td>
</tr>
<tr>
<td>$k_{cat}/K_b$, M$^{-1}$s$^{-1}$</td>
<td>(1.9 ± 0.3) × 10$^7$</td>
<td>(4.4 ± 0.3) × 10$^6$</td>
</tr>
<tr>
<td>$K_i$, mM</td>
<td>0.9 ± 0.3</td>
<td>no$^c$</td>
</tr>
<tr>
<td>$k_{red}$, s$^{-1}$</td>
<td>$\geq 700^d$</td>
<td>60 ± 1</td>
</tr>
<tr>
<td>$K_d$, mM</td>
<td>$\leq 0.05^e$</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

$^a$Enzyme activity was measured at varying concentrations of both D-arginine or D-histidine and PMS in 20 mM Tris-Cl, pH 8.7, at 25 °C. $^b$Kinetic data are readily accounted for with the kinetic mechanism of Scheme 6.2. $^c$Not observed. $^d$Estimated lower limiting value. $^e$Estimated upper limiting value. The reaction occurs within the dead time of the spectrometer, thus cannot be accurately measured by stopped-flow techniques.

6.6 Discussion

**Steady State Kinetic Mechanism.** The steady state kinetic mechanism of D-arginine dehydrogenase has been determined with D-arginine and D-histidine as substrates at pH 8.7 and is consistent with the minimal ping-pong bi-bi mechanism illustrated in Scheme 6.2. After the formation of the Michaelis complex DADH$_{ox}$$ \cdot $S, the amino acid substrate is oxidized to the corresponding imino acid with concomitant reduction of the enzyme-bound flavin (DADH$_{red}$$ \cdot $P). Release of the imino product from the active site of the enzyme completes the reductive half-reaction. The oxidative half-reaction with the artificial electron acceptor PMS occurs through the initial formation of a Michaelis complex DADH$_{red}$$ \cdot $PMS, within which catalysis yields the DADH$_{ox}$$ \cdot $PMSH$_2$ complex. Turnover is then completed with the release of reduced PMS from the oxidized enzyme. Evidence in support of a ping-pong kinetic mechanism comes from the steady state kinetic data determined at varying concentrations of D-arginine or D-histidine and PMS, and from the substrate inhibition of enzymatic turnover at high concentrations of D-
arginine. First, the parallel lines observed in the plots of the reciprocal of the initial rates of reaction as a function of the reciprocal of the substrate concentrations is consistent with the irreversible step of product release ($k_5$) occurring before binding of PMS to the reduced enzyme ($k_7$). D-Arginine inhibition of turnover is readily explained with the formation of a dead-end complex $DADH_{\text{red}}\cdot$Arg when D-arginine at high concentrations binds to the free reduced enzyme (in blue in Scheme 6.2). In agreement with a ping-pong kinetic mechanism where PMS reacts with the reduced enzyme after the iminoarginine product of the reaction is released from the enzyme active site, the $K_{PMS}$ had similar values irrespective of whether D-histidine or D-arginine was the substrate, namely $\sim$10 $\mu$M (Table 6.2).

Scheme 6.2 Proposed Steady State Kinetic Mechanism for the Oxidation of D-Arginine Catalyzed by D-Arginine Dehydrogenase. DADH$_{\text{ox}}$, oxidized D-arginine dehydrogenase; S, substrate; DADH$_{\text{red}}$, reduced D-arginine dehydrogenase; P, imino product; PMSH$_2$, reduced phenazine methosulfate. Arg, D-arginine; $k_5$, $k_{11}$ are shown as irreversible because initial rate are measured in the absence of products; $k_4$, although shown, is close to zero as suggested by stopped-flow kinetic data (see text); $k_9$ is shown as irreversible based on the oxidation-reduction potential of PMS/PMSH$_2$ > FAD/FADH$_2$, with values of +80 mV and -200 mV (60), respectively.

In principle, a kinetic pattern with parallel lines could also be obtained with PMS binding to a $DADH_{\text{red}}\cdot$P complex formed by an irreversible conversion of the DADH$_{\text{ox}}\cdot$S complex (15). If
this were the case, D-arginine inhibition of turnover would require the formation of either a ternary complex of the enzyme with two D-arginines or a quaternary complex of the enzyme, PMS and two D-arginines. Both these possibilities appear very unlikely since the available crystallographic structure of the enzyme co-crystallized with D-arginine shows that the active site cavity of the enzyme is almost entirely occupied with a single iminoarginine, thereby preventing the binding of a second arginine (9). Inhibition of catalytic turnover due to formation of a dead-end complex between the substrate and the reduced form of the enzyme was previously observed in other flavoproteins, such as nitroalkane oxidase (16), aldehyde oxidase (17), flavocytochrome b₂ (18), cellobiose dehydrogenase (19) and thymidylate synthase (20).

**Substrate Binding.** Binding of the D-amino acid substrate to the oxidized enzyme occurs in rapid equilibrium. This establishes that the rate constant for the dissociation of the D-amino acid from the DADHₒₓ•S complex destined for catalysis ($k_2$) must be significantly larger than the rate constant for its oxidation to yield the products of the reaction ($k_3$). Evidence to support this conclusion comes from the solvent viscosity effects on the second-order rate constants for the capture of D-arginine or D-histidine onto the enzyme to yield complexes committed to catalysis ($k_{cat}/K_{Arg}$ and $k_{cat}/K_{His}$) (Figure 6.4). These data are readily explained if one considers that a line with a slope between zero and +1 would be expected in a plot of the normalized $k_{cat}/K_m$ values as a function of increasing relative viscosity of the solvent for a reductive half-reaction whose overall rate was at least partially controlled by the diffusion of the substrate in the active site of the enzyme (21). Such a linear dependence of the normalized $k_{cat}/K_m$ values on solvent viscosity was not observed with D-arginine dehydrogenase, for which the solvent viscosity effect was either negligible with a slope not significantly different from zero in the case of D-arginine or inverse and hyperbolic in the case of D-histidine (Figure 6.4). The lack of a solvent viscosity
effect on the $k_{cat}/K_{Arg}$ value is somewhat surprising because large $k_{cat}/K_m$ values in the range of $10^6 \text{ M}^{-1}\text{s}^{-1}$ like the one determined here with D-arginine are commonly associated with some degree of diffusion-control for the binding of substrates to enzymes (22-25).

Substrate binding to the oxidized enzyme in rapid equilibrium immediately establishes the $K_d$ values determined in the stopped-flow spectrophotometer as the true dissociation constants reporting on the thermodynamic equilibrium of the free oxidized enzyme and the Michaelis enzyme-substrate complex (i.e., $K_d = k_2/k_1$) (Scheme 6.2). Although an accurate determination of the $K_d$ value with D-arginine could not be obtained due to the reaction being too fast to be followed with a stopped-flow spectrophotometer, the rapid kinetic data on the reductive half-reaction suggest a $K_d$ value for D-arginine lower than 50 μM. Thus, binding of D-arginine in the active site of the enzyme is at least 200-fold tighter than that of D-histidine, with a $K_d$ value of 10 mM. Tighter binding of D-arginine to D-arginine dehydrogenase as compared to D-histidine is primarily due to the favorable electrostatic interaction of the guanidinium side chain of D-arginine with the active site residue Glu87; such an interaction is not present with D-histidine, as suggested by the structures of the enzyme in complex with iminoarginine and iminohistidine recently reported (Figure 6.5) (9). The importance of an electrostatic interaction involving a glutamate residue for selective binding of the substrate in the active site of a flavoenzyme was recently established in choline oxidase through mechanistic and structural studies (11). In that case, site directed mutagenesis studies or the use of substrate analogs devoid of charge were consistent with a ~15 kJ mol$^{-1}$ energetic contribution of the side chain of Glu312 to the ionic interaction with the positively charged choline (11). A comparable energetic contribution to substrate binding is likely provided by Glu87 in D-arginine dehydrogenase, as suggested by the at least 200-fold ratio of the $K_d$ values estimated for D-arginine and D-histidine.
**Isomerization of the Michaelis Complex with D-Histidine.** After the initial formation of the Michaelis complex involving the oxidized enzyme and D-histidine, the DADH$_{ox}$•His species isomerizes to produce an enzyme-substrate complex DADH$_{ox}$•His* that is competent for the subsequent reaction of flavin reduction (Scheme 6.3). Evidence for this conclusion comes from the effect of increasing solvent viscosity on the normalized $k_{cat}/K_{His}$ values determined in the presence of glycerol. The $k_{cat}/K_{His}$ values increased hyperbolically to a limiting value with increasing viscosity of the solvent (Figure 6.4), consistent with the presence of an internal equilibrium of the enzyme-substrate complex in the reductive half-reaction. Moreover, in the crystal structure of D-arginine dehydrogenase in complex with iminohistidine, the ligand is present in two conformations with respect to the flavin (Figure 6.5) (9). One of the two conformations presents the imino group of iminohistidine in the same orientation relative to the
flavin 7,8-dimethyl-isoalloxazine that is seen in the crystal structure of the enzyme in complex with iminoarginine (Figure 6.5) (9). This conformation is likely to be competent for catalysis in the enzyme-substrate complex. The alternative conformation most likely is not competent for the subsequent reaction of flavin reduction, as suggested by the relative orientation of groups participating in the hydride transfer reaction. Thus, the isomerization of the DADH<sub>ox</sub>•His complex can be readily explained as reflecting the reversible conversion of multiple binding conformations, not all of which are catalytically competent for the subsequent oxidation reaction involving the flavin. The isomerization of the DADH<sub>ox</sub>•S complex is not observed with D-arginine as substrate. This stems from a more optimal binding mode of D-arginine as compared to D-histidine, which also exploits the extra interaction of the positively charged side chain with the active site Glu87 (vide ante) (Figure 6.5) (9). Isomerizations of Michaelis complexes prior to the reaction of flavin reduction have been previously observed in the wild type and selected mutant forms of flavocytochrome b<sub>2</sub> (26) and in a mutant form of choline oxidase where the active site Glu312 is replaced with aspartate (11).

\[
\text{DADH}_{\text{ox}} \xrightleftharpoons[k_2]{k_1} \text{DADH}_{\text{ox}} \text{-His} \xrightleftharpoons[k_4]{k_3} \text{DADH}_{\text{ox}} \text{-His}^* \xrightarrow[k_5]{P} \text{DADH}_{\text{red}}
\]

Scheme 6.3 Isomerization of the Michaelis Complex with D-Histidine. DADH<sub>ox</sub>, oxidized D-arginine dehydrogenase; His, D-histidine; DADH<sub>red</sub>, reduced D-arginine dehydrogenase; P, iminohistidine.

**Flavin Reduction.** The oxidation of the amino acid substrate catalyzed by D-arginine dehydrogenase entails the two-electron, irreversible reduction of the enzyme-bound flavin without formation of any observable reaction intermediates. Evidence for this conclusion comes from the rapid kinetic data on the reductive half-reaction determined in a stopped-flow spectrophotometer, yielding a monophasic decrease in the absorbance of FAD when the enzyme is mixed with D-histidine as substrate. Irreversibility of the flavin reduction (i.e., \( k_4 \) being close
to zero in Scheme 6.2) is established from the extrapolation to the origin of the hyperbolic dependence of the $k_{\text{obs}}$ values as a function of the concentration of the D-histidine substrate (27). Lack of observable reaction intermediates implies that if they were formed they would have to decay at rates that are at least 20-times faster than their rates of formation. Although alternate mechanisms for flavin reduction cannot be ruled out at this stage, these observations are consistent with the mechanism for substrate oxidation by hydride transfer that has been previously proposed for other flavoproteins acting on amino acids, such as D-amino acid oxidase (28, 29) and sarcosine oxidase (29), $\alpha$-hydroxy acids, such as flavocytochrome $b_2$ (26, 30, 31) or alcohol substrates, such as choline oxidase (32, 33), aryl alcohol oxidase (34), and pyranose 2-oxidase (35).

The oxidation of D-arginine by D-arginine dehydrogenase is at least 20-times faster than the oxidation of D-histidine, as indicated by the rate constants for flavin reduction estimated upon mixing the enzyme and the substrate in the stopped-flow spectrophotometer. The larger $k_{\text{red}}$ value seen with D-arginine likely originates from a better orientation and positioning in the active site of D-arginine with respect to D-histidine, due to the favorable interaction of its side chain with Glu87 (Figure 6.5) (9). The importance of substrate orientation and positioning for efficient oxidation of an organic molecule have been recently established in another flavoprotein, choline oxidase, where it was shown that the conservative replacement of an active site Glu with Asp results in a 230-fold decrease in the $k_{\text{red}}$ value for the oxidation of the alcohol substrate (11).

**Product Release.** With D-arginine as substrate, the release of iminoarginine from the active site of the enzyme is partially rate limiting for the overall turnover of the enzyme. This conclusion is supported by the effect of increasing solvent viscosity on the normalized rate constant for the overall turnover number of the enzyme at saturating concentrations of D-
arginine and PMS (i.e. $k_{\text{cat}}$). The reaction would be slower in solvents with higher viscosities if a diffusion-controlled process is the rate-limiting step (36). Since product release is the only diffusive step during turnover when the enzyme is saturated with substrates, a line with a slope of 0.14 as observed in Figure 6.4 indicates that the release of the product is partially rate limiting in turnover. In contrast, enzymatic turnover with D-histidine is not limited by the rate of product release from the enzyme active site, as suggested by the lack of solvent viscosity effects on the $k_{\text{cat}}$ value with D-histidine. Instead, the overall turnover of the enzyme with D-histidine is at least partially limited by the kinetic step of flavin reduction, as suggested by the comparison of the $k_{\text{red}}$ value of 60 s$^{-1}$ determined by using rapid reaction kinetics and the $k_{\text{cat}}$ value of 35 s$^{-1}$ determined by using the steady state kinetic approach.

**Lack of Oxygen Reactivity.** D-Arginine dehydrogenase is a true dehydrogenase that reacts very poorly, if at all, with molecular oxygen. This conclusion is supported by the lack of oxygen consumption observed upon mixing the enzyme with D-arginine or with 15 other D-amino acids at pH 8.7 as determined by using a Clark-type oxygen electrode. In contrast, an initial rate of $\sim$800 s$^{-1}$ was observed upon addition of 1 mM PMS as an electron acceptor to the same reaction mixture, identifying the enzyme as a true dehydrogenase.

The analysis of the crystal structure of the enzyme and its comparison with that of D-amino acid oxidase, which readily reacts with oxygen (i.e., $k_{\text{cat}}/K_{\text{oxygen}} = 10^5$ M$^{-1}$s$^{-1}$) (37), helps to rationalize the lack of oxygen reactivity in D-arginine dehydrogenase. First, the methyl side chain of Ala46 appears to physically block access of oxygen to the reactive C(4a) atom of the flavin in the active site of D-arginine dehydrogenase (Figure 6.6). The corresponding residue in D-amino acid oxidase is Gly52 (with the numbering of the enzyme from *Rhodotorula gracilis*) (38). When Gly52 is replaced with a bulkier Val the resulting mutant enzyme loses the ability to
react with oxygen due to steric hindrance preventing oxygen access to the reduced flavin (38). The importance of permitting physical access of oxygen to the C(4a) atom of the flavin has also been shown with site-directed mutagenesis in L-galactono-γ-lactone dehydrogenase (39, 40). In the wild type enzyme, which reacts poorly with oxygen, access to the C(4a) atom of the flavin is blocked by the side chain of Ala113. However, replacement of this residue with a smaller Gly yields a 400-fold increase of the $k_{cat}/K_{oxygen}$ to $3.4 \times 10^5$ M$^{-1}$s$^{-1}$, a value that is typically found in oxidases (39).

**Figure 6.6** Active site of D-arginine dehydrogenase (PDB number 3NYE) (9). For clarity, only selected amino acid residues are shown. The FAD cofactor is shown as a stick representation. The side chain of Ala46 is shown in green.

Lack of oxygen reactivity in D-arginine dehydrogenase is also most likely associated with the absence of positive charges in close proximity of the C(4a) and N(1)-C(2) atoms of the flavin. In this regard, the positive charge of the substrate/product of D-arginine dehydrogenase can be immediately ruled out since the ping-pong steady state kinetic mechanism establishes that the reaction of the reduced flavin with the electron acceptor occurs on the enzyme after the release of
the reaction product from the enzyme. The positive charge of Arg305 is the closest to both the C(4a) and N(1)-C(2) atoms of the flavin, at distances \( \geq 7 \text{ Å} \) (Figure 6.6). In some oxidases the N-terminal end of an \( \alpha \)-helix providing a partial positive charge either reinforces or substitutes for the effect of the full positive charge proximal to the flavin N(1)-C(2) atoms, as exemplified by D-amino acid oxidase (41-43), cholesterol oxidase (44), and polyamine oxidase (45). However, in D-arginine dehydrogenase the closest \( \alpha \)-helix is 6 Å away from the N(1)-C(2) atoms of the flavin and points in the opposite direction (Figure 6.6). These positive charges, which are typical in several flavoprotein oxidases (46-52), exert two independent roles. The positive charge close to the flavin C(4a) atom has been proposed to facilitate the stabilization of the negatively charged superoxide species that transiently forms in the reaction of the reduced flavin with oxygen. This has been established in a number of flavoprotein oxidases by using mechanistic and structural approaches, among which are glucose oxidase (53), monoamine oxidase (54, 55), monomeric sarcosine oxidase (56) and choline oxidase (57, 58). The positive charge close to the N(1)-C(2) atoms of the flavin has been proposed to provide electrostatic stabilization of the anionic hydroquinone form of the reduced flavin, which is a common feature in flavoprotein oxidases but not in dehydrogenases (59).

**Conclusions.** In summary, we have engineered an untagged form of D-arginine dehydrogenase, purified it to high levels and investigated its steady state kinetic mechanism and reductive half-reaction with the substrates D-arginine and D-histidine. The results of the kinetic investigation show that the enzyme is a true dehydrogenase that does not react in its reduced state with molecular oxygen. With both amino acid substrates the enzyme displays a ping-pong bi-bi kinetic mechanism when PMS is used as electron acceptor. Flavin reduction is partially rate limiting for the overall turnover of the enzyme with the slow substrate D-histidine but not with
the fast substrate D-arginine. With the latter, release of the iminoarginine product of the oxidation of D-arginine from the active site of the enzyme is partially rate limiting for the overall turnover of the enzyme. An isomerization of the Michaelis complex is also established with D-histidine prior to the flavin kinetic step, likely reflecting the conversion of multiple binding conformations that are not all catalytically competent for the subsequent flavin reduction. The kinetic investigation of the authentic untagged form of D-arginine dehydrogenase reported herein, along with the recent structural investigation of the iminoarginine- and iminohistidinenzyme complexes at high resolutions, will provide a solid framework for future mutagenesis and mechanistic studies aimed at the characterization of the enzyme.

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CHAPTER 7

On the Catalytic Mechanism of D-Arginine Dehydrogenase

7.1 Abstract

The chemical mechanism of leucine dehydrogenation, catalyzed by D-arginine dehydrogenase (DADH), has been explored with a combination of the effects of pH, substrate, and solvent KIE and proton inventory by using rapid kinetics technique. The effect of pH on the reductive half-reaction shows that DADH binds the zwitterionic form leucine. Values for the rate constant of flavin reduction are strongly-pH dependent indicated that an unprotonated group for conversion leucine to iminoleucine. These results support a hydride transfer mechanism for the dehydrogenation reaction in the DADH. The reductive half-reaction with leucine proceeds via releasing a proton for the substrate with $\alpha$-NH$_3^+$ form, followed by a hydride transfer from C$_{\alpha}$ to N$_5$ flavin.

7.2 Introduction

The oxidation of primary amines plays important roles in biology and is crucial to several biochemical processes, such as neurotransmission (1), cell growth and differentiation (2) and neoplastic cell proliferation (3, 4). The enzymes that oxidize C-N bonds have been shown to utilize NADP$^+$, copper and 2,4,5-trihydroxyphenylalanine quinone (TPQ) or flavins (5-8). Glutamate dehydrogenase serves as the prototype for the NADP$^+$-dependent class of primary amine oxidizing enzymes (5), copper-amine oxidase for the Cu$^{2+}$-TPQ enzymes (6) and D-amino acid oxidase and monoamine oxidase for the flavin-dependent family (7, 9, 10) (11).

Amine oxidation in glutamate dehydrogenase has been proposed to proceed through a direct hydride transfer from the C$_{\alpha}$ atom of glutamate to the NADP$^+$. The reaction is triggered by the
enzyme-catalyzed deprotonation of the substrate $\alpha$-NH$_3^+$ group in the active site of the enzyme (12).

Mechanistic studies on copper amine oxidase have elucidated the involvement of a Schiff base of the TPQ with the amino group of the substrate, which remains bound to the reduced cofactor after substrate oxidation (13). The oxidation of the reduced TPQ by molecular oxygen through two one-electron steps and a TPQ semiquinone intermediate complete the turnover of the enzyme (14).

With D-amino acid oxidase the ability of the enzyme to catalyze elimination of HCl from $\beta$-Cl-alanine and of oxidizing this substrate to $\beta$-Cl-pyruvate was initially interpreted in favor of a carbanion mechanism of amine oxidation (Scheme 7.1a) (15). Lack of a suitable base in the active site of the enzyme, however, does not support a carbanion mechanism of catalysis (7, 16, 17). The results of mechanistic studies with physiological substrates and of structural studies with the enzyme in complex with inhibitors are consistent with a hydride transfer mechanism of catalysis (7, 16, 17). Lack of solvent kinetic isotope effect (KIE) and an inverse $^{15}$N isotope effect of 0.994 on the $k_{cat}/K_m$ value with D-serine as substrate are consistent with a hydride transfer mechanism from the C$_\alpha$ atom of the anionic amine to the flavin N(5) atom (Scheme 7.1b) (18, 19). What promotes the formation of the anionic amine in the enzyme-substrate complex is not clear, although the lack of a suitable active site base was taken as independent evidence that the active form of the substrate must be the anionic amino acid (7, 16, 20). Computational studies have also supported a hydride transfer mechanism from the anionic amino acid to the flavin rather than a carbanion mechanism (21). With monoamine oxidase the inactivation of the enzyme by trans-2-phenylcyclopropylamine was initially used to propose that oxidation of primary amines involves radical intermediates (Scheme 7.1c) (22-25). A
nucleophilic mechanism for amine oxidation in which a flavin C(4a) adduct is formed prior to proton transfer from the substrate Cα atom to the flavin N(5) atom (Scheme 7.1d) was also proposed (26, 27)(28). However, the lack of detectable intermediates in stopped-flow reductive half-reactions (29, 30) with physiological substrates and the insensitivity of the rate constants for enzyme reduction by benzylamines (31) make radical and nucleophilic proposals in monoamine oxidase not conclusive. Thus, the mechanism for primary amine oxidation catalyzed by flavin-dependent enzymes has been controversial for several decades.

(a) Carbanion mechanism

(b) Hydride transfer mechanism

(c) Radical mechanism

(d) Nucleophilic mechanism
We have recently sub-cloned, expressed and purified to high levels a novel member of the class of flavin-dependent, amine-oxidizing enzymes, namely D-arginine dehydrogenase (DADH) (32, 33). The enzyme from *Pseudomonas aeruginosa* catalyzes the flavin-linked oxidation of D-arginine to iminoarginine, which is nonenzymatically hydrolyzed to 5-guanidino-2-oxopentanoic acid and ammonia (Scheme 7.2). DADH is a strict dehydrogenase, in that during turnover its reduced FAD reacts with electron acceptors other than molecular oxygen (33), presumably ubiquinone. The enzyme displays broad substrate specificity, being able to oxidize all D-amino acids except for D-glutamate, D-aspartate and glycine (32, 34). In the crystal structure of the enzyme in complex with iminoarginine resolved to 1.3 Å the side chain of Glu87 forms a strong ionic interaction with the guanidinium group of the ligand (Figure 7.1) (32), providing a firm rationale for D-arginine being the best substrate with a $k_{cat}/K_m$ value of $10^6$ M$^{-1}$s$^{-1}$ (33). Substrate accessibility to the active site is proposed to be controlled by a mobile loop composed of residues 50-56, which assumes two distinct conformations in the ligand-free and product-bound crystallographic structures (32). The hydroxyl moiety of Tyr53 points outward to the solvent in the ligand-free conformation and it is 3.3 Å and 4.2 Å away from the carboxylate and imino groups of iminoarginine in the product-bound conformation (32). With arginine as substrate flavin reduction ($k_{red}$) occurs with a rate constant $\geq 700$ s$^{-1}$, precluding mechanistic investigations of the reductive half-reaction catalyzed by the enzyme with this substrate (33). Finally, several
ionic and hydrogen bond interactions are established between the carboxylate of iminoarginine and the side chains of Tyr\textsubscript{53}, Arg\textsubscript{222}, Arg\textsubscript{305} and Tyr\textsubscript{249}, and the main chain O atom of Gly\textsubscript{332}, suggesting that these residues may play roles in substrate binding (Figure 7.1).

![Scheme 7.2 Oxidation of D-Arginine Dehydrogenase by DADH](image)

**Scheme 7.2** Oxidation of D-Arginine Dehydrogenase by DADH

In the present study, the mechanism for amine oxidation catalyzed by DADH was investigated with leucine as substrate by using rapid kinetics and mechanistic probes, such as pH effects, substrate and solvent deuterium KIEs and solvent viscosity effects. D-leucine was chosen as the flavin-reducing substrate because it is the slowest substrate of DADH for which the kinetic
parameters $k_{\text{cat}}$ and $K_{m}$ had been previously determined by using the steady state kinetic approach (32). The mechanistic investigation is consistent with amine oxidation by DADH occurring via a hydride transfer mechanism from an anionic substrate that is formed through the enzyme-catalyzed deprotonation of the zwitterionic substrate. Molecular dynamics analysis of the effect exerted by the enzyme active site on the $pK_a$ value of the $\alpha$-NH$_3$ group of leucine corroborates the experimental findings. The mechanistic conclusions are interpreted in view of the high-resolution structures of DADH reported recently (32).

7.3 Experimental Section

Materials. D-Leucine was from TCI America (Portland, OR). D-Leucine-d$_{10}$ was purchased from CDN Isotopes (Canada). Sodium deuterium oxide (99%) was from Isotec Inc. (Miamisburg, OH). Deuterium oxide (99.9%), glucose and glucose oxidase were obtained from Sigma-Aldrich (St. Louis, MO). Oxidized DADH was prepared as described previously (33). All of the other reagents were of the highest purity commercially available.

Rapid Reaction Kinetics. Reductive half-reactions were carried out using an SF-61DX2 HI-TECH KinetAsyst high performance stopped-flow spectrophotometer, thermostatted at 25 °C. Unless otherwise stated, the observed rate constants for flavin reduction ($k_{\text{obs}}$) were determined at varying concentrations of D-leucine in 20 mM sodium pyrophosphate, pH 10.3, under pseudo-first order conditions where the final concentration after mixing of the enzyme was 10 µM and that of the substrate between 0.5 and 50 mM. The stopped-flow traces obtained anerobically were identical to those obtained aerobically. Leucine and leucine-d$_{10}$ were used as substrates to determine the substrate deuterium KIE. For the determination of the solvent deuterium KIE and the proton inventories in solvents containing varying mole fractions of D$_2$O, the pD values were
adjusted using DCI and NaOD based on the empirical relationship (eq 1) that exists between the pH-meter reading and the pD value at varying mole fractions of D$_2$O (n) (35). Solvent viscosity effects were measured in the presence of 9% glycerol as viscosigen, in both the tonometer containing the enzyme and the syringes containing the organic substrates. The resulting relative viscosity at 25 °C was 1.26, which is slightly above the value of 1.23 representing a 100% solution of D$_2$O (36). The pH dependence of the reductive half-reaction of the DADH was studied in the stopped-flow as described above in the pH range from 7.0 to 11.0. Sodium phosphate (20 mM final) was used in the pH range from 7.0 to 8.0; sodium pyrophosphate was used in the pH range from 8.5 to 10.3 and EDTA was used in the pH range from 10.5 to 11.0. Instability of the enzyme precluded extending the pH studies at pH values lower than 7.0.

\[
(\Delta pH)_n = 0.076n^2 + 0.3314n
\]  

(1)

**Steady State Kinetic Parameters.** The steady state kinetic parameters were determined by measuring initial rates of reaction with an oxygen electrode at varying concentrations of leucine and a fixed, saturating, concentration of 1 mM for PMS as electron acceptor as previously described (33). All reaction rates are expressed per molar concentration of enzyme-bound flavin.

**Data Analysis.** Data were fit with the KaleidaGraph software (Synergy Software, Reading, PA) and the Hi-Kinetic Studio Software Suite (Hi-Tech Scientific, Bradford on Avon, U. K.).

Time-resolved flavin reductions were fit to eq 2, which describes a single exponential process for flavin reduction; \(k_{\text{obs}}\) represents the observed first-order rate constant associated with the absorbance changes at 449 nm at a given concentration of substrate, \(t\) is time, \(A\) is the absorbance at 449 nm at any given time, \(B\) is the amplitude of the absorbance change, and \(C\) is the absorbance at infinite time.

\[
A = B\exp(-k_{\text{obs}}t) + C
\]  

(2)
Reductive half-reaction parameters were determined by using eq 3, where \( k_{\text{obs}} \) is the observed first-order rate constant for the reduction of the enzyme-bound flavin at any given concentration of substrate \( (S) \), \( k_{\text{red}} \) is the limiting first-order rate constant for flavin reduction at saturating concentrations of substrate, and \( K_d \) is the apparent dissociation constant for the dissociation of the enzyme-substrate complex that undergoes flavin-reduction into free enzyme and substrate.

\[
k_{\text{obs}} = \frac{k_{\text{red}} S}{K_d + S}
\]  

(3)

Substrate deuterium KIE were calculated using eq 4, which applies for a KIE on the \( k_{\text{red}} \) value. Solvent deuterium and multiple KIESs were calculated using eq 5, which describes a KIE on the \( k_{\text{red}} \) value and a solvent effect on the \( K_d \) value. \( F_i \) is the fraction of heavy atom, \( Dk_{\text{red}} \) is the KIE on \( k_{\text{red}} \), and \( D(K_d) \) is the KIE or, as in the case under study here (see Results), a solvent effect due to pH on the apparent \( K_d \) value.

\[
k_{\text{obs}} = \frac{k_{\text{red}} S}{(K_d + S)[1 + F_i(Dk_{\text{red}} - 1)]}
\]  

(4)

\[
k_{\text{obs}} = \frac{k_{\text{red}} S}{K_d[1 + F_i(Dk_{\text{red}} - 1)] + S[1 + F_i(Dk_{\text{red}} - 1)]}
\]  

(5)

The pH dependence of the \( k_{\text{red}} \) value was determined by fitting the data with eq 6, where \( k_{\text{red}}(\text{lim}) \) is the limiting, pH-independent rate constant for flavin reduction at high pH, and \( pK_a \) is the apparent value for the ionization of a group that must be unprotonated for flavin reduction.

\[
\log k_{\text{red}} = \log \frac{k_{\text{red}}(\text{lim})}{10^{-pK_a}} \frac{10^{-pH}}{10^{-pK_a}}
\]  

(6)

The pH dependence of the \( K_d \) value was determined by fitting the data with eq 7, which describes a curve with a slope of +1 at high pH and a plateau region that defines a limiting, pH-independent \( K_d \) value at low pH, i.e., \( K_d(\text{lim}) \).
\[ \log(K_d) = \log(K_{d\text{ (lim)}} \cdot (1 + 10^{pH - pK_d})) \]  

(7)

The proton inventory on the \(k_{\text{red}}\) value was fit with eq 8 (35). Here, \((k_{\text{red}})_n\) is the limiting first-order rate constant for flavin reduction in \(n\) mole fraction D\(_2\)O, \((k_{\text{red}})_{H_2O}\) is the limiting first-order rate constant for flavin reduction in H\(_2\)O, and \(\Phi_T\) is the isotopic fractionation factor of the transition state proton.

\[ \frac{(k_{\text{red}})_n}{(k_{\text{red}})_{H_2O}} = 1 - n + n\Phi_T \]  

(8)

7.4 Results

**Time-resolved Flavin Reduction.** The time-resolved reduction of DADH was investigated in a stopped-flow spectrophotometer at pH 10.3 and 25 °C by monitoring the loss of absorbance of the oxidized flavin at 449 nm upon mixing the enzyme with the reducing substrate leucine. Pseudo-first order conditions with ~10 μM enzyme and 0.5-50 mM reducing substrate were maintained, and the stopped-flow traces could be fit to single exponentials to obtain observed rate constants \((k_{\text{obs}})\) for flavin reduction at any given substrate concentration (Figure 7.2A). The \(k_{\text{obs}}\) value was hyperbolically dependent on the concentration of the reducing substrate (Figure 7.2B), allowing for the determination of the limiting rate constant for flavin reduction \((k_{\text{red}})\) and the apparent equilibrium constant for the dissociation of the substrate from the ES complexes that will undergo flavin reduction \((K_d)\). The best fit of the data was obtained with the curve extrapolating to the plot origin (Figure 7.2B), consistent with flavin reduction being irreversible, and in agreement with previous results with histidine as the reducing substrate (33).
**Figure 7.2** Effects of pH on the kinetic parameters of RHR (solid circle) and SSK (empty circle) for DADH with leucine as the reducing substrate. Panel A shows the pH profile of $k_{\text{red}}$ and $k_{\text{cat}}$. Data were fit to eq. 5, yielding $k_{\text{red}}(\text{lim}) = 133 \pm 5 \text{ s}^{-1}$ and $pK_a = 9.6 \pm 0.1$ ($R^2 = 0.999$). Panel B shows the pH profile of $K_d$ and $K_m$. Data were fit to eq. 6, yielding $K_d(\text{lim}) = 3.5 \pm 0.3 \text{ mM}$ and $pK_a = 10.3 \pm 0.1$ ($R^2 = 0.89$). Panel C shows the pH profile of $k_{\text{red}}/K_d$ and $k_{\text{cat}}/K_m$. Data were fit to eq. 7, yielding $k_{\text{red}}/K_d(\text{lim}) = 16,000 \pm 2,000 \text{ M}^{-1}\text{s}^{-1}$ and $pK_a$ values of $9.2 \pm 0.1$ and $11.0 \pm 0.2$.

**pH Effects on Flavin Reduction.** The effects of pH on the $k_{\text{red}}$ and $K_d$ values for DADH with leucine as the reducing substrate were determined in the pH range 7.0-11.0. This was carried out with the goal of determining the ionization states and the apparent $pK_a$ values of protein groups that participate in the reaction of amine oxidation. At all pH values tested, and ensuring that pseudo-first order conditions were maintained, the kinetics of the reductive half-reaction followed the general trends of **Figure 7.2**, allowing us to establish $k_{\text{red}}$ and $K_d$ values as a function of pH. The $k_{\text{red}}$ increased with increasing pH and reached a plateau at high pH with a limiting value of $133 \pm 5 \text{ s}^{-1}$ (**Figure 7.3A**). An apparent $pK_a$ of $9.6 \pm 0.1$ for an unprotonated group participating in flavin reduction was established. The pH profile of the $k_{\text{red}}$ value also established that solvent KIEs are devoid of artifactual contributions that originate from pH effects at $pL \geq 10.3$, i.e., where the $k_{\text{red}}$ is pH-independent. The $K_d$ was pH-independent between below pH 9.5 and increased with increasing pH values, defining an apparent $pK_a$ of $\sim 10.3$ for a group that must be protonated for substrate binding to yield ES complexes that will undergo flavin reduction (**Figure 7.3B**).
**Figure 7.3** Effects of pH on the kinetic parameters of RHR (solid circle) and SSK (empty circle) for DADH with leucine as the reducing substrate. Panel A shows the pH profile of $k_{\text{red}}$ and $k_{\text{cat}}$. Data were fit to eq. 5, yielding $k_{\text{red}}(\text{lim}) = 133 \pm 5 \text{ s}^{-1}$ and $pK_a = 9.6 \pm 0.1 \ (R^2 = 0.999)$. Panel B shows the pH profile of $K_d$ and $K_m$. Data were fit to eq. 6, yielding $K_d(\text{lim}) = 3.5 \pm 0.3 \text{ mM}$ and $pK_a = 10.3 \pm 0.1 \ (R^2 = 0.89)$. Panel C shows the pH profile of $k_{\text{red}}/K_d$ and $k_{\text{cat}}/K_m$. Data were fit to eq. 7, yielding $k_{\text{red}}/K_d(\text{lim}) = 16,000 \pm 2,000 \text{ M}^{-1}\text{s}^{-1}$ and $pK_a$ values of $9.2 \pm 0.1$ and $11.0 \pm 0.2$.

**Substrate Deuterium KIE on $k_{\text{red}}$.** The substrate deuterium KIE on the $k_{\text{red}}$ value was determined at pH 10.3 using leucine-$d_{10}$ and leucine as the reducing substrates, to report on the status of the substrate CH bond in the transition state(s) for the amine oxidation catalyzed by the enzyme. In aqueous solution the $^D(k_{\text{red}})_{\text{H2O}}$ had a value of $5.1 \pm 0.1 \ (\text{Figure 7.4})$, consistent with cleavage of the substrate CH bond being manifested in the transition state for the reaction of flavin reduction. As expected, there was no KIE associated with the $K_d$ value and the best fit of
the data was obtained by using eq 4. Upon substitution of water with deuterium oxide there was a small, but significant, decrease in the magnitude of the KIE with a $D(k_{\text{red}})_{D^2O}$ value of $4.7 \pm 0.1$ ($R^2 = 0.9996$).

**Figure 7.4** Observed rate constants as a function of substrate concentration. Black curve represents substrate reduction of DADH enzyme with leucine as a substrate in H$_2$O. Red curve represents substrate reduction of DADH enzyme with leucine as a substrate in D$_2$O. Blue curve represents substrate reduction of DADH enzyme with leucine-d10 as a substrate in H$_2$O. Data were fit to eqs 4 and 5.

**Solvent Deuterium KIE on $k_{\text{red}}$.** The solvent deuterium KIE on the $k_{\text{red}}$ value was determined at pH 10.3, where the $k_{\text{red}}$ value is pH-independent (Figure 7.3A), to report on the status of bonds involving solvent exchangeable protons in the transition state(s) for the amine oxidation catalyzed by the enzyme. A normal $D^2O(k_{\text{red}})_H$ value of $1.77 \pm 0.01$ ($R^2 = 0.9995$) was determined with leucine as substrate (Figure 7.4). The best fit of the data was obtained by using eq 5, which allowed also for a solvent effect on the $K_d$ value of $2.8 \pm 0.1$ to be computed. Such a solvent effect on the $K_d$ value is primarily, if not entirely, due to pH because $K_d$ is not pH-independent at pH 10.3 (Figure 7.3). For this reason, the solvent effect on the $K_d$ value was not considered.
further in this study. Upon replacing leucine with leucine-d₁₀ there was a small, but significant, decrease in the D₂O(ᵣᵣₑｄ) value to 1.60 ± 0.04 (R² = 0.9989).

As a control that the solvent effect on ᵣᵣₑｄ was not due to the increased viscosity of D₂O as compared to H₂O, the ᵣᵣₑｄ value was determined in aqueous solutions in the absence and presence of 9% glycerol, which is isoviscous with D₂O (36, 37). At pH 10.3, the ᵣᵣₑｄ was similar when determined in the presence and absence of glycerol, with a (ᵣᵣₑｄ)H₂O/(ᵣᵣₑｄ)glycerol ratio of 1.03 ± 0.03. These results established that the solvent deuterium KIE on the ᵣᵣₑｄ value reports on solvent exchangeable proton(s) being in flight in the transition state for the reaction of flavin reduction.

**Proton Inventory on ᵣᵣₑｄ.** The proton inventory technique was used to gain insights on the number of solvent exchangeable protons responsible for the solvent deuterium KIE observed in the time-resolved reduction of DADH. The ᵣᵣₑｄ value was determined at pH 10.3 in solutions with varying mole fractions of deuterium oxide, and the data were analyzed in a plot of the reciprocal of the KIE as a function of the mole fraction D₂O (Figure 7.5) (38). The resulting ᵣᵣₑᵈ proton inventory was linear, suggesting that a single solvent exchangeable proton is in flight in the transition state for the reaction of flavin reduction (Figure 7.5).

![Figure 7.5 Proton inventory for the reduction rate, ᵣᵣₑᵈ, using leucine at pH 10.3.](image)
Multiple Deuterium KIEs on $k_{\text{red}}$. Multiple deuterium KIEs were determined at pH 10.3 to elucidate whether the substrate and solvent deuterium KIE determined independently on the $k_{\text{red}}$ value for DADH occurred in the same or in multiple kinetic steps. The $^{D,\text{D}_2\text{O}}k_{\text{red}}$ value, representing the effect of substituting leucine in H$_2$O with leucine-d$_{10}$ in D$_2$O, was 7.8 ± 0.1 (calculated by using eq 5). Such a multiple deuterium KIE is lower than the product of the individual substrate and solvent KIEs, i.e., $^{D}(k_{\text{red}})_{\text{D}_2\text{O}} \times^{D_2\text{O}}(k_{\text{red}})_{\text{H}}$, yielding a computed value of 9.0 ± 0.2. This is consistent with the substrate and solvent KIEs on flavin reduction occurring not in the same transition state (39).

pH Effects on the Steady State Kinetic Parameters. Previous results with arginine or histidine as substrate for DADH indicated that the enzyme follows a Ping-Pong Bi-Bi steady state kinetic mechanism (33). Moreover, irrespective of the amino acid substrate the $K_m$ value for PMS, which is used as electron acceptor, is ~10 μM (33). This establishes that the apparent steady state kinetic parameters determined at a fixed concentration of 1 mM PMS, i.e., ~100-fold larger than $K_m$, approximate well the true $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ values for the amino acid substrates. Consequently, the effects of pH on the steady state kinetic parameters were determined here by measuring initial rates of reaction with leucine as the varying substrate for DADH at a fixed concentration of 1 mM PMS. A high non-enzymatic reactivity of PMS with oxygen seen at alkaline pHs prevented to extend the profile above pH 10.0. Nonetheless, the pH-profile of the $k_{\text{cat}}$ value was similar to that of the $k_{\text{red}}$ (Figure 7.3A), with the $k_{\text{cat}}$ being between 1.2- to 1.6-times lower than the $k_{\text{red}}$ throughout the pH range considered (Table S7.2). This establishes that flavin reduction primarily contributes to limiting the overall turnover of the enzyme with leucine as substrate. The $K_m$ values between pH 7.0 and 10.0 were remarkably
similar to the $K_d$ values determined by using a stopped-flow spectrophotometer (Figure 7.3B),
indicating that with leucine as substrate the $K_m$ value reports directly on the apparent affinity of
the enzyme for substrate.

7.5 Discussion

In this paper, deuterium KIEs have been used to obtain sights into the reductive half-reaction
mechanism of leucine oxidation in the reaction catalyzed by DADH. The results of the rapid-
reaction kinetic analyses described here are consistent with the kinetic mechanism of Scheme
7.1b. Strong evidence for such a mechanism comes from the substrate, solvent deuterium kinetic
IEs and proton inventory determined on the reductive half-reaction presented here. The oxidation
of leucine catalyzed by DADH involves deprotonation of amino group of the substrate and
hydride transfer from $\alpha$-hydrogen of the substrate to the N5 of the flavin. Two bonds cleavage
occurs in an asynchronous fashion. The substrate KIE on $k_{\text{red}}$ provides a direct probe of the status
of the CH bond, whereas the solvent KIE on $k_{\text{red}}$ can be used to provide information on the status
of the NH bond.

The oxidation of leucine requires an unprotonated group for catalysis, as suggested by the
effects of pH on the rate constant for flavin reduction. The $k_{\text{red}}$ values of DADH exhibited pH
dependence with limiting value of $133 \pm 5$ s$^{-1}$ at high pH, and a $pK_a$ of about 9.6 was measured
for group that must be unprotonated for efficient catalysis. Based on the crystal structure and
kinetic data, the $pK_a$ of 9.6 was assigned to the residue Tyr53 which stabilize the partially
positively charged $\text{NH}_2^+$ group of transition-state. From the DADH-iminoarginine complex
structure, in the product-bound conformation, the aromatic ring of Tyr53 moves into the active
site and close to the imino group of the imino acid (32). Therefore, at high pH, the deprotonated
Tyr53 will form the electrostatic interaction with imino group and stabilize the transition-state
which facilitates the reductive half-reaction. Tyrosine residues are generally found to be conserved and have similar function in other flavin-dependent enzymes. The residue 224 in pig kidney D-amino acid oxidase was suggested to participate in the reductive half-reaction and could stabilize the second charge-transfer intermediate, interacting with the positively charged NH$_2^+$ group of the product imino acid (40). Tyr254 in flavocytochrome b2 was proposed to take part in Michaelis complex formation and stabilize transition-state (41). The kinetic properties of Tyr129 glycolate oxidase provide evidence that the main function of the hydroxyl group of tyrosine is to stabilize the transition state (42).

DADH binds the protonated amino group of leucine, as suggested by the pH profile of the $K_d$ values. From the pH profile, the substrate affinity decrease while increasing the pH, defining an apparent pK$_a$ of ~10.3. Therefore, there is a protonated group involved in substrate binding. The pK$_a$ can be assigned to the substrate amino group. At high pH, the binding affinity is decreased. This indicates that DADH binds the zwitterionic form of leucine, the predominant species in solution. Alternatively, the pK$_a$ of 10.3 can also be assigned to Tyr249. Based on the previously reported crystal structure of enzyme-iminoarginine complex (32), there are several electrostatic and hydrogen bond interactions between iminoarginine and the side chain or main chain atoms of Tyr53, Glu87, Arg222, Tyr249, Arg305, and Gly332. These residues constitute the site of interaction for binding substrate. Due to the pK$_a$ value is around 10.3, two tyrosines are good candidates. Since Tyr53 forms H-Bond with amino group of the substrate, deprotonation will not affect $K_d$. Therefore, the pK$_a$ of 10.3 was assigned to the residue Tyr249. The increasing in $K_d$ at high pH was due to the deprotonation of Tyr249 which forms hydrogen bond with carboxyl group of the product complex. Since there is only one pK$_a$ observed in pH profile of $K_d$, the pK$_a$ of the amino group should either less than 7.0 or large than 11.0. Computational studied
investigated by Hamelberg’s group shows the pK_a of amino group of the substrate will increase while the substrate binds to the enzyme (personal communication). In this case, the pK_a of substrate amino group is larger than 11. Therefore, the DADH still binds the zwitterionic form of leucine at the pH used in the experiment. The observation that the anionic form of leucine with a neutral amino group is the reactive species in the reduction is similar to that observed for other flavoenzymes, such as monomeric sarcosine oxidase (43), monoaime oxidase (22, 44) and trimethylamine dehydrogenase (45, 46).

The hydride ion transfer from substrate to the enzyme-bound flavin and proton abstraction from the amine substrate are both rate-limiting steps in the overall turnover of the DADH enzyme. This conclusion is supported by both steady state kinetics and rapid kinetic data. First, the pH-profile of the \( k_{\text{cat}} \) value was similar to that of the \( k_{\text{red}} \), with the \( k_{\text{cat}} \) being between 1.2- to 1.6-times lower than the \( k_{\text{red}} \) throughout the pH range considered. Independent evidence comes from the \( Dk_{\text{cat}} \) value with deuterated leucine significantly larger than unity, with \( D(k_{\text{red}})_{H_2O} \) value of 5.1 ± 0.1 and \( D(k_{\text{red}})_{D_2O} \) value of 4.7 ± 0.1. Thus, the CH bond cleavage is almost fully rate-limiting step. In the reaction catalyzed by DADH enzyme the abstraction of the amino proton is associated with deuterium kinetic isotope effect significantly larger than unity, with \( D2O(k_{\text{red}})_{H} \) value of 1.77 ± 0.01. According to the lack of viscosity effect, the observed solvent isotope effect is consistent with the cleavage of substrate N-H bond. Moreover, linear relationship observed in the proton inventory associated with \( k_{\text{red}} \) values suggests a single proton being in flight in the catalysis. Thus, the proton form substrate amine group can be removed by the active site residue or solvent. Therefore, the overall turnover of the enzyme is limited primarily by the substrate C-H bond cleavage with minimal contribution of substrate NH bond cleavage.
Timing of CH bond and NH bond cleavage. An asynchronous mechanism is proposed for CH bond and NH bond cleavage based on the kinetic isotope effect determined on the rate constant for flavin reduction. Since in the asynchronous mechanism, upon slowing down the cleavage of the other bond a decreasing in the magnitudes of the substrate and solvent kinetic isotope effects is expected. The decrease in the rate constants from the substrate and solvent kinetic isotope effect were observed upon replacing H₂O with D₂O and leucine with deuterium leucine, respectively. Consistent with asynchronous mechanism, the multiple kinetic isotope effects $^{D,D_2O}k_{red}$ value of $7.8 \pm 0.1$ were smaller than the product of the individual substrate and solvent kinetic isotope effects $(^D(k_{red})_{D_2O} \times ^{D_2O}(k_{red})_{H})$, with a value of $9.0 \pm 0.2$.

There are forward and reverse commitments to catalysis of the enzyme-substrate complex at pH 10.3. This conclusion is supported by the asynchronous mechanism for the cleavage of NH and CH bonds of the substrate. Since the $k_{cat}$ being 1.41-times on average lower than the $k_{red}$ throughout the pH range, the $k_{cat}$ value at pH 10.3 can be estimated about $81 \pm 3$ s⁻¹ according to $k_{red}$ with a value of $113$ s⁻¹. Thus, based on the eq 9, $k_{NH}$ can be calculated as $290 \pm 10$ s⁻¹. At pH 10.3, the observed substrate and solvent kinetic isotope effects are 5.1 and 1.77, respectively. Therefore, the $^{D_2O}k_{NH}$ and $^Dk_{CH}$ can be calculated with values of 3.7 and 6.7 by eq 10 and 11.

\[
k_{cat} = \frac{k_{NH}k_{CH}}{k_{NH} + k_{CH}}
\]  \(9\)

\[
^{D_2O}k_{red} = \frac{^{D_2O}k_{NH} + k_{NH}}{1 + \frac{k_{NH}}{k_{CH}}}
\]  \(10\)

\[
^Dk_{red} = \frac{^Dk_{CH} + k_{CH}}{1 + \frac{k_{CH}}{k_{NH}}}
\]  \(11\)
Conclusions. In summary, the results of the mechanistic investigation with deuterated substrate and solvent presented in this study indicate substrate NH bond cleavage and CH bond cleavage that corresponds to reduction of the flavin cofactor involves two steps. The oxidation of leucine proceeds via the releasing of a proton from the substrate with α-NH$_3^+$ form. Oxidation this species occurs by an unprotonated group with a pK$_a$ of 9.6 for direct hydride transfer from the amino acid C$_\alpha$ to the flavin.

7.6 Support Information

Table S7.1 Reductive Half-Reaction Kinetic Parameters for DADH with Leu as Substrate.$^a$

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{\text{red}}, \text{s}^{-1}$</th>
<th>$K_{d, \text{leu}}, \text{mM}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.33 ± 0.01</td>
<td>2.6 ± 0.1</td>
<td>0.999</td>
</tr>
<tr>
<td>7.5</td>
<td>0.93 ± 0.01</td>
<td>3.5 ± 0.1</td>
<td>0.999</td>
</tr>
<tr>
<td>8.0</td>
<td>3.5 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>0.995</td>
</tr>
<tr>
<td>8.5</td>
<td>8.4 ± 0.3</td>
<td>3.2 ± 0.4</td>
<td>0.991</td>
</tr>
<tr>
<td>9.0</td>
<td>26 ± 1</td>
<td>4.8 ± 0.4</td>
<td>0.997</td>
</tr>
<tr>
<td>9.5</td>
<td>50 ± 2</td>
<td>4.3 ± 0.5</td>
<td>0.994</td>
</tr>
<tr>
<td>10.0</td>
<td>91 ± 1</td>
<td>6.8 ± 0.2</td>
<td>0.999</td>
</tr>
<tr>
<td>10.3</td>
<td>113 ± 1</td>
<td>9.7 ± 0.3</td>
<td>0.999</td>
</tr>
<tr>
<td>10.5</td>
<td>119 ± 2</td>
<td>9.1 ± 0.3</td>
<td>0.999</td>
</tr>
<tr>
<td>10.7</td>
<td>125 ± 5</td>
<td>15 ± 1</td>
<td>0.997</td>
</tr>
<tr>
<td>11.0</td>
<td>129 ± 4</td>
<td>16 ± 1</td>
<td>0.998</td>
</tr>
</tbody>
</table>

$^a$ Activity assays of DADH were performed in 20 mM buffer pH for 6.0 to 11.0, at 25°C. Kinetic parameters were fit to eq. 2.

Table S7.2 Steady State Kinetic Parameters for DADH with Leu as Substrate$^a$

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{\text{cat}}, \text{s}^{-1}$</th>
<th>$K_{\text{m, D-leu}}, \text{mM}$</th>
<th>$k_{\text{cat}}/K_{\text{m, D-leu}}, \text{M}^{-1}\text{s}^{-1}$</th>
<th>$k_{\text{red}}/k_{\text{cat}}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.26 ± 0.01</td>
<td>3.1 ± 0.2</td>
<td>80 ± 4</td>
<td>1.26 ± 0.06</td>
<td>0.997</td>
</tr>
<tr>
<td>7.5</td>
<td>0.64 ± 0.01</td>
<td>3.6 ± 0.3</td>
<td>180 ± 10</td>
<td>1.45 ± 0.02</td>
<td>0.997</td>
</tr>
<tr>
<td>8.0</td>
<td>2.3 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>870 ± 50</td>
<td>1.54 ± 0.09</td>
<td>0.999</td>
</tr>
<tr>
<td>8.5</td>
<td>7.0 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>2,100 ± 200</td>
<td>1.20 ± 0.06</td>
<td>0.997</td>
</tr>
<tr>
<td>9.0</td>
<td>17 ± 1</td>
<td>4.7 ± 0.6</td>
<td>3,700 ± 500</td>
<td>1.54 ± 0.09</td>
<td>0.993</td>
</tr>
<tr>
<td>9.5</td>
<td>31 ± 1</td>
<td>3.8 ± 0.3</td>
<td>8,200 ± 700</td>
<td>1.61 ± 0.08</td>
<td>0.996</td>
</tr>
<tr>
<td>10.0</td>
<td>73 ± 3</td>
<td>6.6 ± 0.5</td>
<td>11,100 ± 900</td>
<td>1.25 ± 0.05</td>
<td>0.993</td>
</tr>
</tbody>
</table>

$^a$Conditions: 20 mM buffer, pH from 7 to 10 and 25°C.
7.7 References


11. A number of flavoproteins are active on N-methylated amino acids, with oxidation of the bond between the amino N atom and the methyl group rather than the Cα atom. Sarcosine oxidase serves as the prototype for this class of flavin-dependent enzymes, which also includes N-methyltryptophan oxidase, N,N-dimethylglycine oxidase, and NikD. The various proposed mechanisms of amine oxidation that have been considered for this class of flavoenzymes, which are equivalent to those in Scheme 7.1 c-d, have been recently reviewed by Dr. Fitzpatrick (see ref. 8).


28. The nucleophilic mechanism was initially proposed by Hamilton and involved the abstraction of the proton from the substrate Cα atom by an active site base occurring after the initial nucleophilic attack of the substrate N atom to the flavin C(4a) atom (see ref. 27). The recent proposal by Edmondson and coworkers where the flavin N(5) atom accepts the proton from the substrate Cα as the substrate N atom forms the flavin-adduct originates from the lack of an appropriate base in the crystallographic structure of the enzyme (see ref. 26).


of substrate and facilitate catalysis with trimethylamine base, \textit{J Biol Chem} 276, 42887-42892.
CHAPTER 8
General Discussion and Conclusions

The dissertation is focused on flavoenzyme mechanisms and uses choline oxidase and D-arginine dehydrogenase as model systems to gain an understanding of enzyme catalysis. Mechanistic and structural studies have been carried out by using pH and kinetic isotope effects, proton inventories and site-directed mutagenesis on the selected active site residues, as well as X-ray crystallography. These allowed investigating the mechanism of alcohol and amine oxidation. In the choline oxidase, the catalytic role of a serine at position 101 has been addressed which is important to both the activation of the alcohol substrate and the subsequent hydride transfer reaction. In addition, the mechanism of D-arginine dehydrogenase was investigated and discussed to gain more insight of the amine oxidation.

Choline oxidase catalyzes the two-step, four-electron oxidation of choline to glycine betaine, with betaine aldehyde as an intermediate. It has been previously studied using biochemical (1-4), site-directed mutagenic (5-12), structural (13-15), mechanistic (16-20) and computational approaches (21). The results show that the oxidation of choline to betaine aldehyde occurs through a hydride transfer mechanism. Catalysis is triggered by the removal of the hydroxyl proton of choline by an active site base and the hydride was subsequently transferred from the α-carbon of the substrate to the N5 atom of the flavin. In the wild-type enzyme, the abstraction of the hydroxyl proton is fast and occurred in the dead-time of the stopped-flow instrument, i.e. ~2.2 ms. Therefore, the proton abstraction was not observed and there was one phase for the flavin reduction in the stopped-flow experiment.
From the X-ray structure of the wild-type choline oxidase resolved to 1.86 Å, Ser101 is less than 4 Å from the N(5) atom of FAD and within hydrogen bonding distance (i.e., <3 Å) of the oxygen atom of DMSO, an additive that was used in the crystallization experiments (15). In order to probe the role of Ser101, the variants in which the serine was replaced with alanine, threonine, cysteine or valine were prepared by site-directed mutagenesis and investigated with rapid kinetics and steady state kinetics.

Firstly, the steady state, transient kinetic and crystal structure studies were used to investigate Ser101Ala variant of choline oxidase. This work suggests that the OH group of Ser101 facilitates the reduction of the enzyme-bound flavin by choline and betaine aldehyde but impedes the reaction with O2. Compared to the wild-type enzyme, the mutant shows no significant structural differences except for the lack of a hydroxyl group on residue 101 and a more planar configuration of the flavin isoalloxazine ring (22). The serine residue in position 101 optimizes enzyme turnover number with suboptimal oxidation half-reactions and faster reductive half-reactions. Thus, the residue at position 101 is important to balance the oxidative and reductive half-reactions and catalyze the reaction by compromise.

Then the rapid reaction kinetic studies of Ser101Ala, Ser101Thr, Ser101Cys and Ser101Val variants were investigated. Two phases were observed for flavin reduction giving a fast phase and a slow phase which is different from the single phase observed in the wild-type enzyme. Use of stopped-flow spectrophotometry revealed two distinct processes that were ascribed to successive deprotonation and oxidation of bound choline. With solvent isotope effect and substrate isotope effect, the results show that the side chain hydrogen bonding is critical for both proton abstraction and hydride transfer. Greatest insight into the catalytic mechanism is developed by comparing the rate constant ($k_5$) for hydride transfer with hydrophilicity of the side
chain at position 101 in a series of variant enzymes in which serine is replaced by alanine, threonine, cysteine and valine. This work convincingly demonstrates that general polarity as well as hydrogen bonding influenced by residue 101 plays a dominate role in catalysis. This study represents the first instance in which the hydrophilic character of the serine residue proximal to the C4a and N5 flavin atoms in a flavin-dependent enzyme, besides its hydrogen bonding capability, has been shown to facilitate the transfer of the hydride ion from the activated alkoxide to the N5 atom of flavin. The conclusions provide a significant contribution to our developing understanding of side chain participation in catalytic turnover of flavoproteins.

D-arginine dehydrogenase catalyzes the oxidation of D-amino acids to the corresponding imino acids, which are hydrolyzed to keto acids and ammonia. Non-covalently linked FAD is used as a cofactor in catalysis and the native electron acceptor still remains unknown (23). D-arginine dehydrogenase, with L-arginine dehydrogenase, has been shown to be a 2-component amino acid racemase in the D- to L- inversion in Pseudomonas aeruginosa (23).

The steady state kinetic parameters with various amino acids and crystal structures of DADH indicate that the negatively charged side chain of Glu87 is responsible for the high selectivity of DADH for positively charged substrates via electrostatic interactions. The other residues in the active site, such as Tyr53, Met240 and Val242, form hydrophobic walls for specificity of the long aliphatic and unbranched substrate. Therefore, the electrostatic and hydrophobic interactions are responsible for selecting D-arginine and D-lysine as the best substrate of DADH. D-tyrosine, D-phenalanine and D-methionine may form favorable Van der Waals interactions with the hydrophobic wall of the active site as good substrate. Moreover, two distinct conformations with a mobile loop composed of residue 50-56 as observed in the ligand-free and product-bound crystallographic structures substrate is proposed to control substrate accessibility
to the active site. The conformational change of the lid, especially Tyr53, may allow DADH to accommodate bulky residues like D-phenylalanine or D-tryptophan. Thus, the electrostatic and hydrophobic interactions bring the substrate into the active site and the lid switch to closed form. Closing of the lid may facilitate the hydride transfer step by shielding the active site and the FAD from solvent to increase the overall hydrophobicity of the active site. Further insight into the function of this active site lid may be obtained by protein engineering studies on DADH. For example, replacement of Glu87 to a positive charged residue may allow the DADH narrowly selecting for negatively charged D-amino acid substrates. Replacement of Tyr53 with a large size residue, tryptophan, may result in big substrates being unable to bind to the active site. Conversely, replacement of Tyr53 with small size residues may decrease the rate for the hydride transfer since the active site would be more exposed to the solvent.

The steady state kinetic mechanism of D-arginine dehydrogenase was investigated and discussed with D-arginine or D-histidine as a substrate. The kinetic investigations show that with both substrates the enzyme displays a ping-pong kinetic mechanism when PMS is an electron acceptor. Meanwhile, a dead-end complex of the reduced enzyme with high concentration D-arginine yielding substrate inhibition and an isomerization involving multiple conformations of the initial Michaelis complex with D-histidine were proposed. The kinetic data along with the structure with iminoarginine or iminohistidine complex will provide a guideline for future mutagenesis and mechanistic studies aimed at the characterization of the enzyme. The mechanism for amine oxidation catalyzed by DADH was investigated with leucine as substrate by using rapid kinetics and mechanistic probes, such as pH effects, substrate and solvent deuterium KIEs and solvent viscosity effects. Substrate KIE on $k_{\text{red}}$ provide a direct probe of the status of the CH bond, whereas the solvent KIE on $k_{\text{red}}$ can be used to provide information on the
status of the NH bond. The pH profile suggests $k_{\text{red}}$ is pH independent at high pH; in contrast, $K_d$ is pH independent at low pH. Substrate kinetic isotope effect on $k_{\text{red}}$ suggests hydride transfer is at least partially rate-limiting step. The solvent isotope effect on $k_{\text{red}}$, lack of viscosity effect and proton inventory studies indicate that a single proton is in flight in the transition state for the reduction of flavin. The multiple isotope effect on $k_{\text{red}}$ is smaller than the product of individual isotope effect. Based on these results, the mechanism of amine oxidation by DADH is proposed. The study shows that the oxidation of leucine catalyzed by DADH involves deprotonation of amino group of the substrate and hydride transfer from $\alpha$-hydrogen of the substrate to the N5 of the flavin. The cleavages of the two bonds occur in an asynchronous fashion. The mechanistic investigation is consistent with amine oxidation by DADH occurring via a hydride transfer mechanism from an anionic substrate that is formed through the enzyme-catalyzed deprotonation of the zwitterionic substrate. Molecular dynamics analysis of the effect exerted by the enzyme active site on the p$K_a$ value of the $\alpha$-NH$_3$ group of leucine corroborates the experimental findings.

Comparative studies of the DADH crystal structure and the crystal structure of D-amino acid oxidase have been carried out. Based on the definition, the enzyme with oxygen as an electron acceptor and hydrogen peroxide as product are called oxidase. Those using other electron acceptors instead of molecular oxygen are dehydrogenases. Using the oxygen electrode, there is no oxygen consumption determined with DADH. However, the addition of an artificial electron acceptor, phenazine methosulfate (PMS), results in the rapid depletion of oxygen since the reduced PMS is easily oxidized by oxygen. Thus, the DADH is a strict dehydrogenase. The lack of oxygen reactivity in DADH has been interpreted based on the crystal structures. First, the accessibility of oxygen may be blocked by residue Ala46 which is right in front of the C(4a)
atom of flavin, the site of oxygen reactivity in flavin dependent enzymes. In D-amino acid oxidase from *Rhodotorula gracilis*, the corresponding residue Gly52 was investigated by replacing to valine. The study showed the loss of oxygen reactivity because steric hindrance prevents the oxygen from reacting with the reduced flavin. However, in L-galactono-γ-lactone dehydrogenase, the Ala113Gly variant showed a 400-fold increase in $k_{cat}/K_{m,ox}$ compared to the wild-type enzyme. The oxygen reactivity is very similar to the typical of oxidases reinforcing the necessity of physical access of oxygen to the reduced flavin. Thus, based on similarities in the positioning of alanine residues in both the enzymes, the lack of oxygen reactivity in DADH can due to the physical barrier provided by the Ala46 residue. Therefore, it would be interesting to replace the residue, glycine, to a smaller residue and study the oxygen reactivity.

The positive charges in the proximity of the C(4a) or N(1)-C(2) atoms of flavin are also important for oxygen reactivity. This has been established in a number of flavoprotein oxidases, such as glucose oxidase (24), choline oxidase (1, 25) and monoamine oxidase (26, 27). However, in the crystal structure of DADH, the positive charged residue or N-terminal end of an α-helix are all far away from this region. Also, the ping-pong steady state kinetic mechanism rule out the positive charged substrate/product since the product will be released before the electron acceptor binds to the active site.

Overall, in this dissertation, the studies on choline oxidase and D-arginine dehydrogenase have provided insight into the oxidation of amines or alcohols by flavoenzymes. The importance of a hydrophilic residue with capability of hydrogen bonding proximal to the C(4a)-N(5) flavin locus in a flavin-dependent enzyme has been established and represents the first instance in which the hydride ion transfer is affected by the character of the side chain. The high-resolution crystal structures as well as the kinetic data have highlighted the important structure differences
that rationalize the catalytic activities and substrate specificity of the enzymes. The steady state kinetic and hydride transfer mechanistic studies will provide useful information on the mechanistic properties on the amine oxidation by other flavin-dependent enzymes.

8.1 References


