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INACTIVATION OF CHOLINE OXIDASE BY IRREVERSIBLE INHIBITORS OR STORAGE CONDITIONS

by

JANE V. HOANG

Under the Direction of Giovanni Gadda

ABSTRACT

Choline oxidase from *Arthrobacter globiformis* is a flavin-dependent enzyme that catalyzes the oxidation of choline to betaine aldehyde through two sequential hydride-transfer steps. The study of this enzyme is of importance to the understanding of glycine betaine biosynthesis found in pathogenic bacterial or economic relevant crop plants as a response to temperature and salt stress in adverse environment. In this study, chemical modification of choline oxidase using two irreversible inhibitors, tetranitromethane and phenylhydrazine, was performed in order to gain insights into the active site structure of the enzyme. Choline oxidase can also be inactivated irreversibly by freezing in 20 mM sodium phosphate and 20 mM sodium pyrophosphate at pH 6 and -20 ^{o}C. The results showed that enzyme inactivation was due to a localized conformational change associated with the ionization of a group in close proximity to the flavin cofactor and led to a complete lost of catalytic activity.

INDEX WORDS: Choline Oxidase, Flavoproteins, Enzyme Inactivation, Chemical Modification, Phenylhydrazine, Tetranitromethane, Mechanism-based Inhibitors, Hysteresis.

INACTIVATION OF CHOLINE OXIDASE BY IRREVERSIBLE INHIBITORS OR STORAGE CONDITIONS

by

JANE V. HOANG

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

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Jane V. Hoang
2006

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By

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Georgia State University
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Chapter I

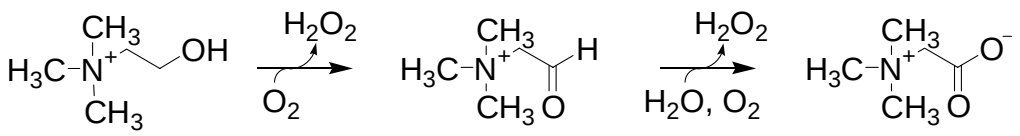
INTRODUCTION

1. Previous Studies of Choline Oxidase

1.1. Early studies. In 1937, an active form of choline oxidase was first isolated from rat liver and kidney by Bernheim and Bernheim (*1*). The enzyme was capable of oxidizing choline to betaine aldehyde with the uptake of one and two moles of oxygen at pH 6.7 and 7.8, respectively. Subsequent work by Mann et *al.* reported a more detailed study on the same enzyme (*2*). In that study, choline oxidase was considered as a typical dehydrogenase due to the fact that rat liver extracts could reduce sodium ferricyanide rapidly under anaerobic conditions. Kinetic studies showed evidence of this enzyme oxidizing choline and arsenocholine, and oxygen uptake was observed corresponding to the formation of aldehyde species. The oxidation of choline and arsenocholine by choline oxidase was inhibited by ammonium ions, trimethylammonium ions, and betaine, suggesting that the trimethylammonium headgroup is important for the binding of the ligand to the enzyme. Since choline dehydrogenase, in the presence of choline and arsencholine, reduced cytochrome c at room temperature without the help of enzyme cofactors, it was proposed that the choline oxidase system was composed of choline dehydrogenase, cytochrome c, and cytochrome oxidase.

Choline oxidase from *Arthrobacter globiformis* was first purified by Ikuta et *al.* in 1977 (*3*). The purification procedure involved fractionations with ammonium sulfate and acetone, followed by column chromatography using the anionic exchanger DEAE-cellulose and gel filtration through a Sephadex G-200 column. The molecular mass of the isolated enzyme was between 71 kDa and 83 kDa, as suggested by SDS-PAGE and gel filtration, respectively. UV-visible absorbance spectra of the enzyme showed maxima at 363 and 450 nm, consistent with the

spectroscopic properties of a flavoenzyme. Kinetic data indicated that choline oxidase is capable of utilizing both choline and betaine aldehyde as substrates to produce glycine betaine with K_m values of 1.2 mM and 8.7 mM, respectively. In addition, the enzymatic activity decreased significantly when a number of substrate analogs were used, suggesting high substrate specificity of the enzyme toward choline and betaine aldehyde. During the catalytic reaction of choline oxidase, formation of hydrogen peroxide was detected as equivalent to the amount of oxygen being consumed. Based on these data, the authors proposed a mechanism for the catalytic reaction by choline oxidase, which involved the oxidation of choline to glycine betaine with betaine aldehyde as the intermediate and molecular oxygen as the electron acceptor (Scheme 1.1) (*3, 4*).



Scheme 1.1. Reaction catalyzed by choline oxidase.

1.2. Prosthetic group of choline oxidase. In 1979, the prosthetic group of choline oxidase from *A. globiformis* was studied in detail by digesting the enzyme into peptide fragments with trypsin and chymotrypsin (*5*). The peptides were purified to reveal a fragment with a typical UV-visible absorbance spectrum of a flavin. The flavin species in choline oxidase was identified as FAD rather than FMN due to the fact that the flavin fluorescence intensity increased by 2-fold upon release of AMP after the hydrolysis of the enzyme with nucleotide pyrophosphatase. When the fluorescence excitation spectrum of the flavin peptide from choline oxidase was compared with that of free riboflavin, a 25 nm hypsochromic shift of the 346 nm peak to 361 nm was observed for the aminoacyl flavin. This suggested that the flavin cofactor

was covalently attached to the enzyme via the 8α-methylene group of its isoalloxazine ring. In order to determine whether the flavin is bound to N(1) or N(3) position of the histidyl ring, sodium borohydride was used to reduce flavin. This was done because the N(1) isomer of histidyl flavin could be reduced in the presence of borohydride, as followed by the quenching of flavin fluorescence, whereas the N(3) isomer cannot be reduced (*6*). The purified flavin peptide of choline oxidase was not reduced by sodium borohydride, suggesting that the flavin cofactor was bound to the N(3) position of the histidine residue. A subsequent study by Ohta-Fukuyama et. *al.* on choline oxidase from *Alcaligens sp.* confirmed 8α-[N(3)-histidyl]-FAD as the prosthetic group of the enzyme, as suggested by the electrophoretic mobility of the hydrolysate of the methylated histidylflavin (*7*). In that study, the flavin was bound to the enzyme with a 1:1 ratio to give a spectrum with 358 nm and 453 nm maxima and a shoulder at 480 nm. The covalently bound FAD in the native enzyme could be reduced with choline or betaine aldehyde under anaerobic conditions and re-oxidized when exposed to air.

The spectroscopic properties of choline oxidase from *Alcaligens sp.* were investigated thoroughly by Ohta et *al.* to provide an in depth understanding of the properties of the covalently bound flavin (*8*). Upon reduction of the enzyme using dithionite or photo-irradiation in the presence of EDTA, formation of flavin semiquinone was observed. This semiquinone was catalytically inert towards organic substrate and oxygen. The insensitivity of the flavin semiquinone toward oxygen makes choline oxidase unique because such feature was not found among other flavoenzymes. Choline oxidase could form a complex with glycine betaine, the product of its catalytic reaction, with a dissociation constant of 17 mM at pH 7.5. When the enzyme-product complex was reduced with dithionite or photo-irradiation under anaerobic conditions, no semiquinone formation was detected. The reduced form of enzyme-product

reacted with oxygen and reoxidized at two different rates: rapid and slow. The authors proposed the formations of two different flavin species in the photoreaction of the enzyme-product complex: a C4a adduct that reacted slowly with oxygen, and an N5 adduct that reacted quickly with oxygen.

In a recent study, Rand et *al.* investigated the flavin linkage in choline oxidase from *A. globiformis* (*9*). In that study, a purified histidylriboflavin was treated with $NaBH_4$ in order to distinguish between the N(1) and N(3) isomers. Treatment of $NaBH_4$ stemmed from the fact that flavin fluorescence of N(1)-histidylriboflavin can be irreversibly quenched upon reduction with $NaBH_4$, whereas N(3)-histidylriboflavin is unreactive toward $NaBH_4$ (*10*). N(1) and N(3) isomers can also be distinguished by their stability in aqueous solution at room temperature: N(1) is stable under these conditions but N(3) can be decomposed to 8-formylriboflavin (*11*). These results, along with the pK_a value from the pH-dependent fluorescence of the histidylriboflavin, indicated an N(1)-linked flavin. The position of the histidine residue to which the flavin is covalently bound was also examined by subjecting choline oxidase to tryptic cleavage. The FAD-containing peptide was isolated using HPLC and analyzed with mass spectrometry. The flavin-containing peptide was found to be the tripeptide His-Ala-Arg at position 87-89 based on the nucleotide sequence of *codA* gene, which has been previously reported in Genbank (accession number X84895) for choline oxidase from *A. globiformis* (*12*). The authors concluded that choline oxidase contains 8-[N(1)-histidyl]flavin at position 87 in its polypeptide chain.[1]

---

[1] Sequencing analysis of the cloned choline oxidase by our group in 2004 showed that the nucleotide sequence of *codA* gene previously reported in Genbank in 2003 by Rand et *al. (12)* contained seven flaws, resulting in a translated protein with significantly altered amino acid sequence between position 298 and 410. Consequently, the results obtained based on that sequence might not be accurate.

1.3. Biotechnological and medical applications. In the past decade, the biotechnological and medical applications of choline oxidase have been studied extensively. This was motivated by the finding that glycine betaine accumulated in the cytoplasm of cells as a resistance for adverse environments with low and high temperature or hyperosmotic stress (*13, 14*). For plants that do not synthesize glycine betaine, such as *Arabidopsis thaliana*, transformation of betaine-synthesizing gene (*codA* gene from *A. globiformis*) into the plant cells significantly increased the survival of the plants in high-salt soils (up to 300 mM NaCl) (*15*) or at freezing temperatures (*16*). Over the years, many studies have been conducted in order to use the protective function of glycine betaine to improve the economic production of crop plants, such as tomato (*17*), rice (*18, 19*), and tobacco (*20*). In all cases, the presence of glycine betaine showed a significant increase in cold and salt stress tolerance in growing plants. Accumulation of glycine betaine has also been observed in human pathogens, such as *Staphylocccus aureus* (*21*), *Pseudomonas aeruginosa* (*22*), and *Listeria monocytogenes* (*23*), in resistance to high osmolarities and unsuitable thermal conditions. *S. aureus* is a bacterium commonly found on the skin of a healthy person; it can cause illnesses ranging from minor skin infections to fatal diseases such as pneumonia, meningitis, endocarditis and septicemia (*13)*. *L. monocytogenes* is a food-born pathogen that causes food poisoning and infections in digestive tracts (*21, 23*). *P. aeruginosa* can cause a variety of systemic infections, such as dermatitis, bacteremia, urinary tract infections, respiratory system infections, soft tissue infections, bone and joint infections, and gastrointestinal infections. For these reasons, understanding the mechanism of the biosynthesis of glycine betaine has the potential for the developments of therapeutic agents that can inhibit the growth of these pathogens in the host tissue.

1.4. Cloning and expression of choline oxidase. As the interest for understanding the biosynthesis of glycine betaine in choline oxidase grew, large quantities of the enzyme were needed for biophysical, mechanistic, and structural investigations. In a recent study by our group, the *codA* gene for choline oxidase was cloned from genomic DNA of *A. globiformis* strain ATCC 8010 and expressed in the *E. coli* strain Rosetta(DE3)pLysS (*24*). The enzyme was purified using DEAE-Sepharose column and characterized using mass spectrometry. The resulting enzyme contained a mixture of the anionic semiquinone and oxidized forms of FAD. The oxidized form of choline oxidase was obtained after 24 h of dialysis at pH 6 and 4 ^{o}C. Gel filtration and mass spectrometry indicated that the enzyme was a homodimer with covalently bound flavin and a molecular mass of 120 kDa. Enzyme assay with choline and betaine aldehyde showed that the enzyme was active and used both as substrates. A newly amino acid sequence was obtained for choline oxidase, showing a greater similarity between choline oxidase and the members in GMC oxidoreductase superfamily than the sequence originally reported in GeneBank (*9*). (See last paragraph in Section 1.2 for more detail)

1.5. Spectroscopic properties of choline oxidase. In another study, the spectroscopic properties of recombinant choline oxidase with its cofactor in the oxidized and semiquinone forms were characterized (*25*). The UV-visible absorbance spectrum of the oxidized enzyme showed two typical flavin peaks at 359 and 452 nm, with an extinction coefficient at 452 nm of 11.4 mM^{-1} cm^{-1} at pH 8. This enzyme form emitted light at 530 nm when being excited at 452 nm. Upon incubation with dithionite under aerobic conditions, the peak at 452 nm in UV-visible absorbance spectrum was bleached to give rise to a new spectrum with maxima at 372 and 495 nm. This spectrum remained unchanged after gel filtration was used to remove excess dithionite, suggesting that choline oxidase stabilized the anionic semiquinone species of the flavin in the

presence of oxygen. Consistent with this observation, the maxima of the flavin UV-visible absorbance spectrum returned to 350 and 452 nm when the enzyme was unfolded with 4 M urea. The circular dichroic spectrum of the enzyme-bound semiquinone showed a sharp negative peak at 406 nm and a broad negative peak from 450 to 650 nm, which was in agreement with the previously reported circular dichroic properties of enzyme-bound anionic flavin semiquinones (*7, 26*). The anionic flavin semiquinone in choline oxidase was converted to its oxidized form with k_{obs} of 219 x 10^{-6} s^{-1} when the enzyme was incubated at pH 6 and 15 ^{o}C. The evidence for this came from the bleaching of the 372 nm peak to give rise to a 452 nm peak in the UV-visible absorbance spectrum. A similar bleaching of the 452 nm peak followed by an increase in absorbance at 320 nm was also observed when the enzyme was treated with sodium sulfite, an indication of the formation of a covalent *N*(5)-flavin adduct. The enzyme bound sulfite with a K_d value of ~50 μM at pH 7 and 15 ^{o}C; such high affinity suggested the presence of a positive charge near the N(1)C(2)=O locus of the flavin. UV-visible absorbance and steady state kinetics were used to monitor the enzyme turnover with either choline or betaine aldehyde. The results showed that the flavin semiquinone remained spectroscopically and kinetically insensitive to the substrates and therefore did not participate in catalysis. The pH dependence of the kinetic parameters was determined for both choline and oxygen using enzyme with oxidized flavin and enzyme containing a mixture of oxidized and semiquinone flavins. The data indicated the presence of a catalytic base with a pK_a of ~ 7.5 that was required for the oxidation of the substrate but not for the reduction of oxygen. In agreement with this observation, an ionizable group with the same pK_a value was required to be protonated for product inhibition, suggesting that this ionizable group might belong to the same amino acid that acted as the catalytic base in the oxidation of substrate.

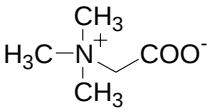
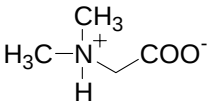
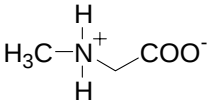
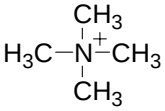
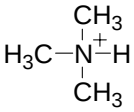
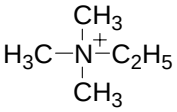
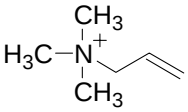
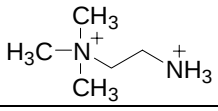
1.6. Kinetic properties of choline oxidase. The kinetic mechanism of commercially available choline oxidase from *A. globiformis* was studied in detail using either choline or betaine aldehyde as substrate (*27*). At pH 7, the steady state kinetic mechanism of the enzyme was determined to be sequential. The reduced enzyme was found to react with oxygen before the second oxidation step or before the release of the product. Based on the kinetic pattern of 1/rate versus $1/[O_2]$, a K_m value of ≤20 μM was estimated for betaine aldehyde as substrate, consistent with betaine aldehyde preferentially partitioning forward to catalysis rather than dissociating from the enzyme (*28*). This conclusion was further supported by the observation that ≤10% betaine aldehyde was found in the reaction mixture under enzymatic turnover with saturating concentrations of choline. The k_{cat} for betaine aldehyde was two times higher than the value observed with choline, suggesting that the oxidation of choline to the aldehyde intermediate may be partially rate-limiting for catalysis. The results obtained from this study led to a proposed mechanism for the reaction catalyzed by choline oxidase, which involved two sequential flavin-linked hydride transfers from choline and the resulting betaine aldehyde intermediate to molecular oxygen.

In a subsequent study, pH and deuterium kinetic isotope effects were used to examine the oxidation of choline to betaine aldehyde (*29*). At atmospheric oxygen, the k_{cat} and k_{cat}/K_m values of choline oxidase increased with increasing pH to limiting values, suggesting the presence of a catalytic base in catalysis. When choline was used as substrate in a pH-dependent study, an apparent pK_a of 7.5 was determined for the catalytic base. This pK_a shifted to 8.0 when choline was substituted with [1,2-$^2H_4$]-choline, suggesting a significant commitment to catalysis. The $^D(k_{cat}/K_m)$ determined in atmospheric oxygen decreased from a limiting value of 12.4 at low pH

to a limiting value of 4.1 at high pH, consistent with the cleavage of C-H bond of choline being fully rate-limiting at low pH.

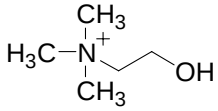
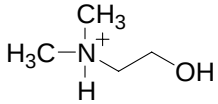
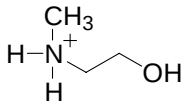
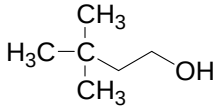
1.7. Mechanistic studies. To determine the substrate recognition and specificity of choline oxidase, choline and glycine betaine analogs were used as substrates and inhibitors, respectively (*30*). As shown in Table 1, the competitive inhibition constant (K_{is}) decreased with increasing number of methyl groups on the substrate. In contrast, replacement of the acetate moiety of glycine betaine with neutral groups, such as methyl, ethyl, allyl, and 2-aminoethyl, did not affect the binding of the ligand to the enzyme. This was suggested by the lack of changes in the K_{is} value. These data indicated that the trimethylammonium moiety is important for substrate recognition and specificity, but the acetate moiety is not. Additional evidence supporting the importance of the trimethylammonium head group of the ligand was found when *N,N*-dimethylethanolamine and *N*-methylethanol were used as substrate analogs for choline. Steady state kinetic data showed that the enzyme was capable of utilizing these compounds as substrate but with a significant decrease in the k_{cat} and k_{cat}/K_m values (Table 2). When 3,3-dimethyl-butan-1-ol was used as substrate, k_{cat} and k_{cat}/K_m values decreased 10 times as compared to those with choline as substrate. This suggested that the positive charge at the trimethylammonium head group was also important for substrate binding.

Table 1.1. Inhibition studies of choline oxidase at pH 6.5[a]

Inhibitor	Structure	K_{is}, mM	R^2
Glycine betaine		15 ± 2	0.984
N,N-Dimethylglycine		57 ± 1	0.995
N-Methylglycine		405 ± 2	0.998
Tetramethylamine		11 ± 1	0.997
Trimethylamine		2.4 ± 0.2	0.998
Trimethylethylamine		13 ± 2	0.988
Allyltrimethylamine		15 ± 1	0.999
2-Amino-trimethylethylamine		9 ± 1[b]	0.993

[a]Choline oxidase activity was measured at varying concentrations of both choline and inhibitor in air-saturated 50 mM sodium pyrophosphate, pH 6.5 and 25 °C, modified from Ref (*30*). [b]Value determined for the protonated form of 2-amino-trimethylethylamine.

Table 1.2. Steady state kinetic parameters for choline and *N*-substituted choline analogs as substrates for choline oxidase at pH 8[a]

Substrate	Structure	k_{cat}, s^{-1}	k_{cat}/K_m[c], $M^{-1}s^{-1}$	R^2
Choline		86 ± 1	200,000 ± 10,000	0.995
N,N-Dimethylethanolamine		5.9 ± 0.1	18,400 ± 300	0.996
N-Methylethanolamine		0.37 ± 0.01	710 ± 10	0.994
3,3-Dimethyl-1-butanol		8.0 ± 0.1	7,900 ± 980	0.980

[a]Choline oxidase activity was measured at varying concentrations of organic substrate and oxygen in 50 mM sodium pyrophosphate, pH 8, and 25 °C, data from Ref. (*30*). [b]k_{cat}/K_m is the k_{cat}/K_m value for the organic substrate.

A subsequent study using pH and solvent viscosity effects with 3,3-dimethyl-butan-1-ol, the isosteric analog of choline, was carried out to support the importance of the positive charge on the trimethylammonium headgroup to substrate binding in choline oxidase (*31*). The pH-dependent study of k_{cat} and k_{cat}/K_m values of choline oxidase with 3,3-dimethyl-butan-1-ol yielded pK_a values that are comparable to the pK_a values previously determined for choline oxidase with choline as a substrate. However, the limiting values of the pH profiles for k_{cat} and k_{cat}/K_m were ~3 and ~400 times lower than those with choline, suggesting a significant decrease in the efficiency of catalytic reaction. This is consistent with k_{cat}/K_m for oxygen when 3,3-dimethyl-butan-1-ol was used as substrate being independent with a limiting value that was ~75 times lower than when choline was used. Solvent viscosity effects were used to examine whether the kinetics steps of substrate binding limit the rate of substrate oxidation steps. As a result, there was no viscosity effects observed for both k_{cat} and k_{cat}/K_m, consistent with the

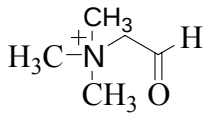
chemical steps in substrate oxidation being fully rate-limiting for the overall turnover of the enzyme with 3,3-dimethyl-butan-1-ol as substrate. Overall, the data presented in this study agreed with previous proposed roles for the positive charge head group on choline being essential for substrate binding and flavin oxidation.

Recently, an in-depth study of the catalytic steps in the reaction catalyzed by the recombinant choline oxidase was carried out in our laboratory using steady state and pre-steady state kinetics approaches under saturating oxygen (*32*). In that study, substrate and solvent isotope effects were used to probe the status of the substrate OH and CH bond cleavage. The results indicated that the reductive half-reaction that involves the cleavage of the substrate CH bond was not masked by other kinetic steps, as suggested by the pH independent value of ~10.6 for $^{D}(k_{cat}/K_{m})$ and a $^{D}k_{red}$ value of 8.9 at pH 10 for the anaerobic reduction of the bound-flavin by choline in both protio and deuterated solvents. At pH 10, no solvent kinetic isotope effect was observed for k_{cat}/K_{m} and k_{cat}, and $^{D}k_{cat}$ and $^{D2O}k_{cat}$ values were 7.3 and 1.1, respectively. These results indicated that the removal of the substrate hydroxyl proton and the hydride transfer from the substrate occur in a stepwise fashion with the formation of a transient alkoxide intermediate species. Overall, the data from both steady state and pre-steady state kinetics provided evidence for the chemical steps of the substrate oxidation by choline oxidase being fully rate-limiting for both the overall turnover and the reductive half reaction involving the oxidation of choline to betaine aldehyde.

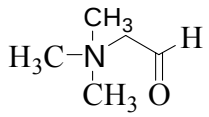
The first oxidation step in the reaction catalyzed by choline oxidase was further characterized by varying oxygen concentrations and temperature in the studies of the kinetic isotope effects with deuterated choline (*33*). The $^{D}k_{cat}$ and $^{D}(k_{cat}/K_{m})$ values were pH–independent at saturating oxygen concentrations, but at ≤0.97 mM oxygen, these values

decreased at high pH to a limiting value. The same pattern was observed for k_{cat} and k_{cat}/K_m when choline was used as substrate at oxygen concentrations ≤0.25 mM. These data suggested that at high concentrations of oxygen, the partition forward to catalysis of the reduced form of enzyme-betaine aldehyde complex was more favored than its reverse partition to the oxidized enzyme-choline alkoxide species. When temperature was varied under saturating oxygen concentrations, $^{D}(k_{cat}/K_m)$ was 10.6 ± 0.6 and temperature independent. The isotope effect on the preexponential factor was determined to be 14 ± 3, which was too large for the classical over-the barrier behavior for hydride transfer, whose preexponential factor was predicted to be between 0.7 and 1.7. In addition, similar enthalpies of activation, $\Delta H^{\ddagger}$, were determined for choline and 1,2-[$^{2}H_4$]-choline, suggesting the cleavage of CH and CD bond did not follow the theoretical model of H and D tunneling, which happen just below the transistion state. That is because this model suggests that the lack of temperature dependence in the kinetic isotope effect is associated with the lack of temperature dependence of the reaction rates with both H and D (*34*). In this study, the similarities in preexponential factor values of k_{cat}/K_m and $^{D}k_{cat}/K_m$ as well as in $\Delta H^{\ddagger}$ for k_{cat}/K_m values with choline and 1,2-[$^{2}H_4$]-choline implied that the tunneling of the hydride observed for the case of choline oxidase may occurs quantum mechanically in a highly preorganized active site, in which environmental vibrations shorten the distance between the donor and the acceptor of the hydride allowing a more efficient hydride transfer (*34-36*).

The mechanism of oxidation of betaine aldehyde was also studied by using spectroscopic and kinetic methods (*37*), in which the steady state and pre-steady state kinetics of choline oxidase with betaine aldehyde and its analogue 3,3-dimethylbutyraldehyde were examined. (It should be noted that 3,3-dimethylbutyraldehyde has the similar chemical structure as betaine aldehyde but without the positive charge at the trimethylammonium head group [Figure 1.1]).



betaine aldehyde



3,3-dimethylbutyraldehyde

Figure 1.1. Chemical structures of betaine aldehyde and 3,3-dimethylbutyraldehyde.

At saturating oxygen, the pH dependence of the kinetic parameters for betaine aldehyde suggested that a catalytic base with a pK_a value of ~6.7 was required for the oxidation of betaine aldehyde. Under anaerobic conditions, choline oxidase was completely reduced in the presence of choline or betaine aldehyde at pH 8 with a k_{red} value of ≥48 s^{-1}. However, only 10-26% of the enzyme was reduced in the presence of 3,3-dimethylbutyraldehyde between pH 6 and 10. Competitive inhibition was observed when enzyme activity was assayed with choline in the presence of 3,3-dimethylbutyraldehyde. NMR spectroscopic analysis revealed that 99% of betaine aldehyde exists in its hydrated form in aqueous solution, while 3,3-dimethylbutyraldehyde exists predominantly in its nonhydrated form. The reason that hydrated form of betaine aldehyde is more favored stems from the stabilization of the hydroxyl group by the positive charge at the trimethylammonium head group. In related work, kinetic data with NAD^+-dependent yeast aldehyde dehydrogenase indicated that the *keto* species of 3,3-dimethylbutyraldehyde was reactive toward enzymic nucleophiles. The data in this study suggested that betaine aldehyde was required to be in its hydrated form for the oxidation to glycine betaine. This was proposed to be a catalytic strategy that allowed choline oxidase to catalyze two sequential oxidation reactions of an alcohol and an aldehyde within the same active site and minimize the amount of energy spent for similar catalytic strategies.

In a search for the catalytic base of choline oxidase, His466 was substituted by both alanine and asparatate (*38, 39*). The choice of mutation at His466 site in choline oxidase

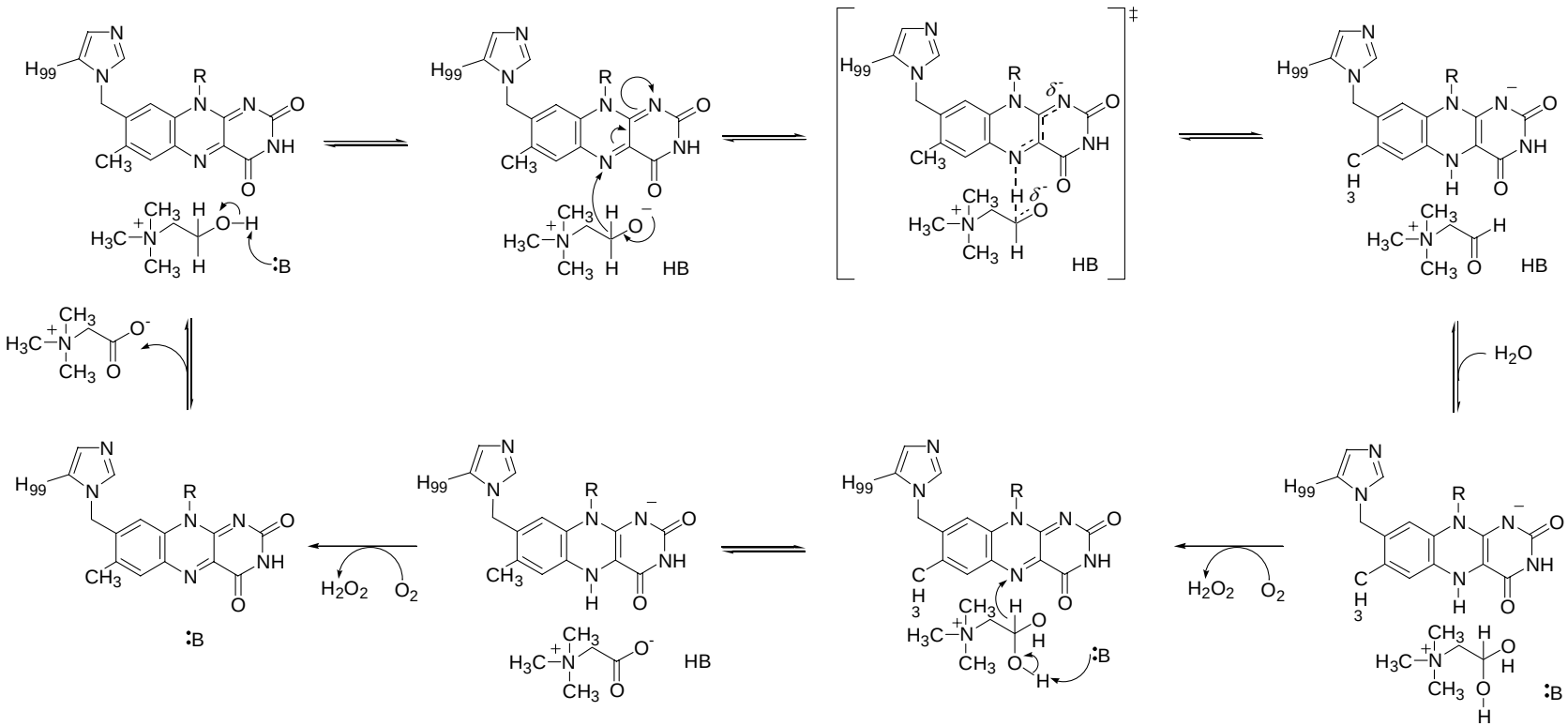
stemmed from previous studies of other enzymes that catalyze the oxidation of alcohol substrates. Based on the studies of cholesterol oxidase (*40, 41*), cellobiose dehydrogenase (*42*), and glucose oxidase (*43, 44*), a histidine is fully conserved within the active site of the enzyme family, corresponding to His466 in choline oxidase, and this residue might act as the catalytic base in the oxidation of alcohol substrates. If His466 were the catalytic base in the reaction, the enzyme would be expected to be completely devoid of activity upon replacing His466 with an alanine. However, the kinetic data for the His466Ala mutant indicated otherwise (*38*). The enzyme was still active with this mutation, but the k_{cat} and k_{cat}/K_m for choline as substrate at atmospheric oxygen decreased 60- and 1000-fold, respectively, as compared to the wild type enzyme. In contrast, the k_{cat}/K_m for oxygen was unaffected, suggesting the involvement of His466 in the oxidation of choline but not in the reduction of oxygen. The UV-visible absorbance spectrum of His466Ala mutant as purified contained the oxidized form of the flavin, whereas wild type choline oxidase as purified contained a mixture of oxidized flavin and its anionic semiquinone (*24, 25*). Treatment of the His466Ala mutant with dithionite under aerobic conditions at pH 8 resulted in the formation of the anionic flavin semiquinone, which was rapidly returned to its oxidized form upon removal of excess dithionite by gel filtration. These data indicated that upon replacing His466 with an alanine, the enzyme lost its ability to stabilize the semiquinone form of flavin in the presence of oxygen. When the mutant protein was incubated with 25 or 100 mM sodium sulfite for 3.5 h at pH 6.5, no change in the absorbance spectrum was detected. This was another difference from the established spectroscopic properties of the wild type enzyme, suggesting that the microenvironment of the flavin was significantly altered upon mutating His466. This conclusion was also supported by the fact that the midpoint reduction potential of two-electron transfer in the catalytically competent-enzyme substrate complex at pH

7 was ~25 mV more negative than wild type enzyme. In the presence of the protonated imidazole, the enzyme activity was partially rescued. In contrast, the neutral imidazole had no effect. These data indicated that His466 in wild type enzyme was protonated. Both the pH-profiles of product inhibition and of k_{cat}/K_m of choline showed an upward shift of pK_a, consistent with a change in polarity of the active site of the enzyme. The relative timing of OH and CH bond cleavage in substrate oxidation was also affected when His466 was replaced with alanine, as suggested by the data from deuterium, solvent, and multiple kinetic effects. Overall, the data ruled out the hypothesis of His466 that was the catalytic base in the reaction catalyzed by choline oxidase. However, the positive charge contributed by the His466 is critical for stabilizing the negative charges in the enzyme active site that found on the N(1) position of the flavin and on the hydroxyl group of the substrate.

In another study, His466 was replaced with an asparatate (His466Asp) (*39*). With the reversal of charge in the active site, the enzyme was completely devoid of activity, as indicated by the lack of oxygen consumption and no bleaching of the 452 nm absorbance peak under anaerobic conditions in the presence of 10 mM choline. However, the enzyme was still capable of binding the substrate because there was a 4 nm bathochromic shift of the 452 nm peak that was associated with an increase in absorbance at 375 nm in the UV-visible absorbance spectrum of the flavin in H466D mutant. A K_d of 0.45 ± 0.04 mM was determined for the binding affinity of the mutant protein for choline by monitoring the spectral changes with increasing concentrations of choline. In contrast, the enzyme had lost its ability to bind glycine betaine because there was no spectral change with increasing concentration of this ligand. The mutation also caused 75% of the flavin to be uncovalently bound in choline oxidase. Anaerobic reduction of the enzyme-bound flavin in His466Asp using xanthine and xanthine oxidase at pH 6 resulted

in the formation of neutral hydroquinone, with no stabilization of the semiquinone intermediate. The data presented in this study indicated that upon replacing a positive charge with a negative charge near the N(1) locus of the flavin in choline oxidase, the enzyme has lost its ability to stabilize the negative charges in the active site, which might lead to defective flavinylation of the protein and change in eletrophilicity of the flavin, therefore resulting in loss of enzymatic activity.

1.8. Summary. Based on the available data, a detailed mechanism for the reaction catalyzed by choline oxidase was proposed. The enzyme catalyzes the oxidation of choline to glycine betaine through two sequential flavin-linked hydride transfers with molecular oxygen as the final electron acceptor and betaine aldehyde as the intermediate. In the first oxidation step (Scheme 1.2), choline is converted into its alkoxide form by an active site base with pK_a of ~7.5. A hydride is transferred from the α-carbon of choline to the enzyme-bound flavin cofactor via tunneling pathway. Consequently, the flavin is reduced and betaine aldehyde is formed as the intermediate. In order to re-oxidize the cofactor for the next oxidation step, the enzyme uses an oxygen molecule as the electron acceptor, resulting in the formation of hydrogen peroxide. Betaine aldehyde is hydrated before the next hydride transfer step. The second hydride transfer happens when the active site base abstract a proton from the gem-diol species of aldehyde, resulting in another CH bond cleavage and the formation of a carboxylate product, glycine betaine. The flavin is reduced by accepting the hydride from betaine aldehyde and reoxidized by transferring two electrons to an oxygen molecule. The turnover cycle of choline oxidase is completed when the product is released from the active site as the flavin becomes oxidized.



Scheme 1.2. The catalytic cycle of choline oxidase. Illustrated based on previous studies of choline oxidase.

2. Phenylhydrazine as a Mechanism-based Inhibitor

Hydrazine compounds have been studied extensively as mechanism-based inhibitors of a wide variety of proteins, including heme-containing proteins such as horseradish peroxidase (*45*), lactoperoxidase (*46*), hemoglobin (*47*), myoglobin (*47*), cytochrome P-450 (*48*), catalase (*49*), flavoproteins such as monoamine oxidase (*50*) and trimethylamine dehydrogenase (*51*), and copper proteins such as dopamine *β*–hydroxylase (*52*) and plasma amine oxidase (*53*). The inhibition mechanisms of the enzymes with hydrazines were studied in detail for a few cases, in which enzyme inactivation usually involved the generation of a carbon-centered radical or anion at the active site (*49, 51*). These species were highly unstable and could react with the cofactors or active site residues in the enzyme. For this reason, hydrazines were used in many chemical modification studies with the aim of identifying important residues in the active site. Among many hydrazines, phenylhydrazine was commonly used as one of the inhibitors of mitochondrial monoamine oxidase, an enzyme found in the central nervous system that can breakdown monoamine neurotransmitters (*54*).

2.1. In flavoenzymes. Approaches for investigation the inhibition effects of phenylhydrazine can be illustrated by studies of monoamine oxidase (*50, 55*) and trimethylamine dehydrogenase (*51, 56*). Monoamine oxidase is a flavoenzyme that catalyzes the oxidative deamination of neurologically active amines, e.g., serotonin. Early studies showed that hydrazines competed with the amine substrate for the enzyme active site (*57-60*). Inactivation of the enzyme was proposed to involve oxidative processes (*61-63*) because maximal inhibition could be achieved only in the presence of oxygen (*64*). Patek and Hellerman later conducted a more detailed study on the inactivation of monoamine oxidase by phenylhydrazine (*50*). The inactivation was shown to be complete and irreversible in the presence of oxygen. Under

anaerobic conditions, the flavin cofactor was reduced by phenylhydrazine but could be reoxidized when exposed to air to give an 80% recovery of enzyme activity. This observation suggested the oxidation of the hydrazine inhibitor occurred before enzyme inactivation. A Hammett study, in which the effects of nuclear substitution on the phenylhydrazine were correlated with the rates of hydrazine-induced inhibition, suggested that flavin reduction by phenylhydrazine was rate-limiting in the overall aerobic inhibition process. Spectroscopic data showed that progressive incubation of the enzyme with phenylhydrazine under aerobic conditions could cause an irreversible bleaching of the flavin peak. When inactive enzyme was digested with trypsin, spectral analysis of the peptide containing the flavin cofactor gave evidence for the formation of a flavin adduct. The intermediate phenyldiazene formed after the oxidation of phenylhydrazine was thought to be responsible for the irreversible modification of flavin cofactor.

The inhibitory mechanism of trimethylamine dehydrogenase with hydrazines has also been studied. Trimethylamine dehydrogenase has several properties in common with mammalian monoamine oxidase, including covalently-bound flavin (*65*) and similar responses to a variety of monoamine oxidase inhibitors (*66*). In work by Nagy et *al.* (*51*), the oxidation of phenylhydrazine to the intermediate phenyldiazene was observed under aerobic and anaerobic conditions. After the flavin peak at 454 nm was bleached upon incubation with phenylhydrazine, a flavin species at 360 nm was seen. This flavin species was isolated by proteolytic digestion and analyzed using mass spectrometry. Upon comparison with model compounds, the modified flavin was identified as an arylated flavin. Generally, absorbance at 360 nm is indicative for flavin modification at either the C(4a) or the N(5) position (*67*). The two sites of modification are distinguished by the fact that N(5) flavin adducts are shifted ~30 nm hypsochromatically in 6

M HCl, whereas C(4a) adducts are shifted ~30 nm in the opposite direction (*67*). The spectral properties of the arylated flavin showed that C(4a) was the position of modification (Figure 1.1).

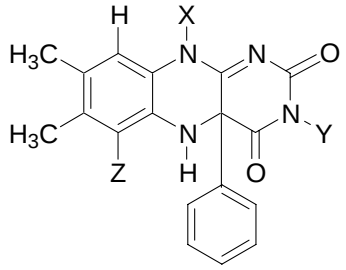
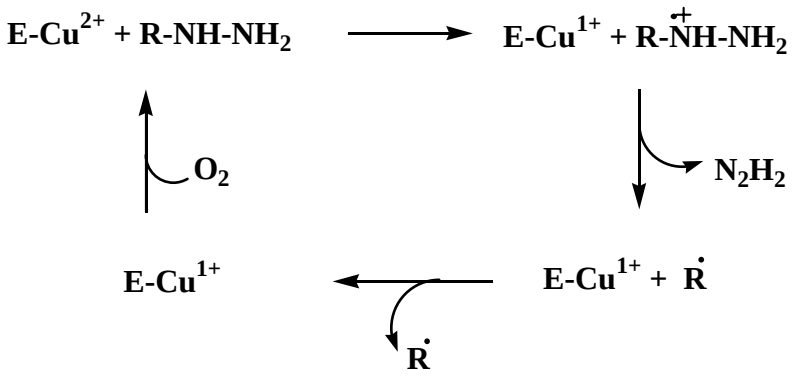


Figure 1.2. Structure of flavin C(4a) adduct. X, Y, and Z represent the side chains on flavin cofactor.

The authors also investigated the reaction of phenylhydrazine and its intermediate phenyldiazene with free flavin. Due to the photosensitivity of free flavin, the experiments were performed in the dark. Phenylhydrazine seemed to react slowly with the flavin, while phenyldiazene reacted rapidly to form the C(4a) adduct. This observation supported the dehydrogenation of phenyhydrazine by the enzyme being a rate-limiting step in the inhibitory mechanism.

2.2. In copper enzymes. The irreversible inactivation by hydrazine inhibitors was also found in copper enzymes. In a study by Fitzpatrick (*52*), dopamine *β*-hydroxylase was inactivated with different hydrazines for the purpose of identifying the active site residues around the copper cofactor. Several hydrazines were tested, including phenyl-, phenethyl-, benzyl-, and methylhydrazine, and hydrazine itself. Enzyme inactivation was observed with phenyl-, phenethyl-, benzyl-, and methylhydrazine, but not with hydrazine. The most rapid inactivation was observed with phenylhydrazine. When the effect of different substrates of dopamine *β*-hydroxylase upon the inactivation rate was tested, the hydrazines were divided into two distinguishable classes. With phenyl-, phenethyl-, and methylhydrazine as inhibitors, the oxidation of copper was required for the inactivation to occur, as suggested by the slower inactivation rate in the presence of the reductant ascorbate. In contrast, the inactivation with

benzylhydrazine required the enzyme in its reduced form, as suggested by the faster inactivation rate in the presence of ascorbate. The substrate tyramine also had different effects on the rate of inactivation when the same inhibitors were used: the inactivation rate decreased with benzylhydrazine, increased with phenylhydrazine and methylhydrazine, but remained unchanged with phenethylhydrazine. In the presence of both tyramine and ascorbate, the inactivation rate decreased in both cases; therefore, the enzyme inactivation might occur at the active site. Such observations were consistent with the proposed inhibitory mechanisms for monoamine oxidase and trimethylamine dehydrogenase discussed above in that the inactivation of the enzyme by hydrazine inhibitors involved the reduction of the enzyme cofactor. To directly test the ability of the enzyme to generate radicals from hydrazines, the α-(-4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (PORN) spin trap was employed to trap the carbon-centered radicals that formed in the reaction. PORN was a reversible inhibitor of dopamine β-hydroxylase, and therefore had already bound to the active site of the enzyme before the radicals were generated, resulting in the trapping of various carbon-centered radicals. Analysis of the PORN-adduct in this study showed that the inhibitory mechanism by monosubstituted hydrazines involved a generation of a hydrazine cation radical, followed by the cleavage of the carbon-nitrogen bond to form a carbon-centered radical and diazene (Scheme 1.2).



Scheme 1.3. Proposed inhibitory mechanism of dopamine β-hydroxylase by monosubstituted hydrazines. Illustration based on the descriptions in ref (*52*).

The generation of a carbon-centered radical from the decomposition of phenylhydrazine radical cation was observed in this study. Based on HPLC analysis, the stoichiometry of labeling of the enzyme was 1 phenylhydrazine per subunit. This was consistent with the presence of one copper in the active site of each monomer. Kinetic isotope effects were also used to gain insights into the catalytic path. When the benzylic hydrogens of benzylhydrazine were replaced with deuterium, an isotope effect of ~13 was observed for V_{max}, and the partition ratio for product formation vs. inactivation decreased up to 11-fold. Therefore, the authors proposed that the inactivation of the enzyme was due to abstraction of an electron from nitrogen instead of abstraction of a hydrogen atom from the benzylic carbon.

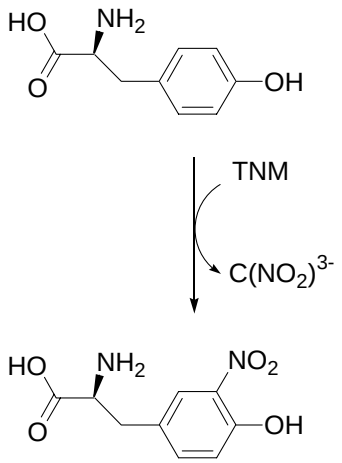
2.3. In heme containing enzymes. The mechanism of inhibition by phenylhydrazine has been studied for heme containing enzymes such as lacrimal-gland peroxidase (LGP) (*68*). LGP is found in the secretion of the lacrimal gland and has the ability to prevent oxidative damage and bacterial infection (*69*). The oxidation of various substrates including halides, pseudohalides, aromatic electron donors and xenobiotics is catalyzed by this enzyme. In a study by Mazumdar et *al.* (*68*), LGP was inactivated irreversibly by the mechanism-based inhibitor phenylhydrazine in the presence of H_2O_2. The enzyme could be protected from the inactivation

in the presence of the electron donor SCN^- and the free-radical trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The experiments with DMPO suggested the involvement of a radical species in the inactivation process. Earlier studies with myoglobin (*70, 71*), hemoglobin (*72, 73*), catalase (*49*), and cytochrome P-450 (*48*) showed the formation of a stable iron-phenyl complex in the interaction of phenylhydrazine with the enzyme. The Soret shift in circular dichroic spectra was used as one of the lines of evidence for the existence of an iron-phenyl complex in the inactive enzyme. In the case of LPG, there was no shift in the Soret region upon treatment with phenylhydrazine. Instead, a significant loss of absorbance intensity at 409 nm and the appearance of a negative ellipticity at 275 nm were observed. The far-UV circular dichroic spectra of the native and modified enzymes were indistinguishable, ruling out inactivation due to a gross structural change upon reaction of radicals with amino acid residues. The authors proposed that the heme iron might be buried deep inside the enzyme where it was inaccessible to the radicals, and the inactivation involved the modification of heme environment. A kinetic study of LPG inactivation showed a stoichiometry of 2:1 of H_2O_2/phenylhydrazine. Therefore, phenylhydrazine was oxidized through one-electron transfer to phenyl radicals, which bound to the vicinity of the heme group, prevented electron transfer from the donor residue to the heme ferryl group, and led to enzyme inactivation.

3. Tetranitromethane (TNM) as an Irreversible Inhibitor

Tetranitromethane (TNM) has been used in chemical modification of many proteins since 1930 (*74*). In many cases, incubation with this reagent resulted in the irreversible inactivation of the enzyme. Detailed studies of the chemical modification using TNM have suggested that TNM is specific for tyrosyl residues (*75, 76*). The reaction of TNM with tyrosine involves the

formation of a charge-transfer complex with the phenol group of the amino acid, followed by the generation of the phenoxide and nitrite radicals due to electron transfer. Nitration occurs when phenoxide and nitrite radicals react, resulting in the release of a nitroformate anion (Scheme 1.4). Additional reactions are possible due to the high reactivity of phenolate radicals with each other.



Scheme 1.4. Nitration of tyrosyl residue by tetranitromethane.

TNM can also oxidize sufhydryl groups under the same conditions (*77*). In a study by Sokolovsky *et al.* (*77*), disulfide and nitrite ions were generated in the presence of excess thiol. Hydrolysis of the intermediate sulfenyl nitrate resulted in the formation of sulfinic acid as the product. The ratio of sulfinic acid to disulfide formation depended on the conditions of the reaction and the nature of the R group on the thiol compounds. A significant extent of other side reactions is also observed for the reaction of enzymes with TNM. However, unwanted reactions can be distinguished by lowering the pH because under acid conditions, i.e. pH $\leq$6, nitration of tyrosine does not occur, whereas the modification is rapid at pH $\geq$9.

Despite the fact that the reaction of enzymes with TNM involves many side reactions, the reagent has been used successfully in identifying active site tyrosines. The most common problem is usually the poor solubility of TNM in water. Therefore, ethanol is usually used to

prepare the TNM stock solutions. The amount of TNM used to modify the enzyme is consequently limited to 10% or less ethanol in the reaction mixture to prevent denaturation of the enzyme by excess ethanol. Chemical modification using TNM is readily quantitated because nitrotyrosine absorbs at 360 nm (Figure 1.3) (*78*).

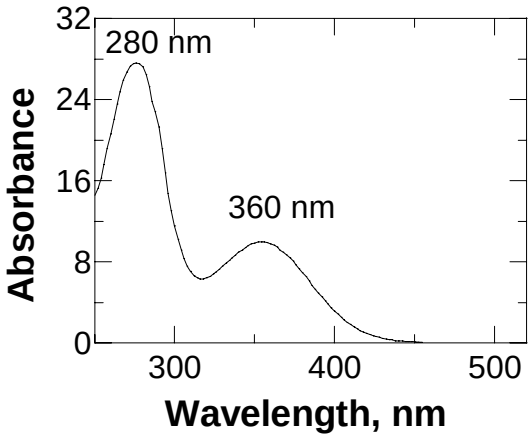


Figure 1.3. Typical spectra of a TNM-modified enzyme. Spectra obtained from the study on chemical modification of choline oxidase with TNM, which described in details in Chapter V.

In a study by Gadda et *al.* (*79*), TNM was used for the modification of the flavoprotein nitroalkane oxidase. Nitroalkane oxidase catalyzes the oxidation of nitroalkanes to aldehydes or ketones with nitrite and hydrogen peroxide as the products. Upon incubation with TNM at pH 7.5, the enzyme was irreversibly inactivated. The rate of inactivation as a function of TNM concentration followed saturation kinetics, suggesting that the inhibitor bound to the enzyme prior to inactivation. In the presence of the catalytic product of the nitroalkane oxidase reaction, the enzyme was protected from inactivation. This indicated that the nitration reaction occurred at the active site of the enzyme. The UV-visible absorbance spectrum of the inactive enzyme showed a significant perturbation in absorbance wavelength as compared to the active enzyme. Comparison of tryptic maps of the treated and untreated enzymes followed by mass

spectrometric analysis revealed a modified tyrosine at position 8[2]. Based on earlier studies of flavoenzymes such as D-amino acid oxidase (*81*), flavocytochrome b2 (*82*), *p*-hydroxybenzoate hydroxylate (*83*), and glucose oxidase (*84*), a conserved tyrosine residue in the active site of these enzymes is found to participate in binding the carboxylate moiety of the substrate. Because the structure of carboxylate group is similar to that of the nitro group, the authors proposed that this tyrosine might also be essential for binding the substrate in nitroalkane oxidase.

4. Effects of Freezing on Enzymes in Storage

For years, research laboratories routinely stored enzymes in frozen solutions because it is believed that the long-term stability of enzymes in frozen solution is greater than in unfrozen solutions. However, in some cases, freezing and thawing may cause substantial enzyme denaturation, which can be detected as alterations of the enzyme structure or loss of catalytic activity. The low temperature perse and pH-shift of the solution as it cools (*85, 86*) usually viewed as the main causes of enzyme denaturation in frozen storage. For this reason, freezing can induce reversible inactivation of pH-sensitive enzymes.

4.1. pH-shift in storage buffers. Although buffers are often used to store enzymes in order to maintain constant pH, evidences for pH shift upon freezing due to precipitation of the less-soluble buffer component have been well documented. For Tris, citrate, and potassium phosphate, the pH has been reported to change up to +1, -0.4, and +0.6 unit, respectively, upon freezing (*87, 88*). The most dramatic pH changes have been observed with disodium phosphate ($Na_2HPO_4.12H_2O$) (*85, 86*) up to 3.5 pH units. In work by Pikal-Cleland et *al.*, protein

[2] With the crystal structure of the nitroalkane oxidase obtained just recently by Nagpal et *al. (80),* the tyrosine that has been identified in the study by Gadda et *al. (79)* was proposed to be at position 398.

denaturation during freezing and thawing in potassium phosphate (KP) and sodium phosphate (NaP) buffer was studied (*86*). Tetrameric *β*-galactosidase was chosen in this study because its inactivation can be monitored with large structural changes from tetramer to monomer. In addition, earlier studies indicated that this protein lost activity when its subunits dissociated at pH ≤ 6 (*89, 90*); the pH optimum for protein stability is between 6 and 8 (*91, 92*). In that study, the pH changes during freezing were determined under nonequilibrium conditions. A combined pH electrode was used with a reference solution containing potassium chloride, glycerol, and formaldehyde that kept the electrolyte solution from freezing and enabled pH measurements down to -30 ^{o}C. When the enzyme was stored at -10 ^{o}C in 100 mM or 10 mM NaP with initial pH of 7.0, the pH significantly decreased to 3.8 or 5.5, respectively. In contrast, when the enzyme was stored in 100 mM or 10 mM KP at -10 ^{o}C and pH 7, the pH of the frozen solutions were 7.3 and 7.1, respectively. The magnitude of pH shifting in both buffers was dependent on buffer concentrations with higher concentrations giving salt crystallization upon freezing. In the ice-liquid interphase, water formed ice crystals and escaped from the buffer phase, resulting in a more solute-concentrated buffer. The precipitation of buffer salts can happen at different times and at various temperatures, depending on their solubility and buffer concentrations (*93, 94*). Ice crystals usually form before the precipitation of buffer salts. These crystals pull the basic phosphate species (i. e., HPO_4^{2-}) away from the solution, thereby inducing a pH shift due to the change in the ratio of base to acid in the buffer. In the case with NaP and KP buffers, disodium salt is much less soluble in water than potassium salt because their eutectic points are -0.5 ^{o}C and -13.7 ^{o}C, respectively. Therefore, the HPO_4^{2-} species of NaP buffer was depleted from solution much earlier than that of KP buffer leading to a greater change in pH. For *β*-galactosidase, the lower recovery of activity and larger structural alterations was observed after freezing and

thawing in NaP buffer than in KP buffer. This was used as evidence for the larger pH shift of NaP. The effects of the freezing and thawing rates on activity recovery of the enzyme were also investigated using different cooling and warming methods. Data on activity recovery and infrared spectroscopy of the enzyme structure revealed that less enzyme was denatured when fast cooling and fast warming methods were used. This implied that rapid freezing and thawing can minimize the time of exposure of the enzyme to low pH and high salt concentrations, thereby resulting in less denaturation of enzyme from storage.

4.2. Reversible inactivation of enzymes. In limited cases, freezing can induce reversible inactivation of pH-sensitive enzymes; this has been observed in the case of L-amino acid oxidase (LAAO) from *Crotalus adamanteus* (*95*). LAAO is a snake venom enzyme that catalyzes a flavin-dependent reaction involving the oxidative deamination of a number of L-amino acids. In that study, the enzyme was stored in 0.2 M Tris-HCl, pH 7.8, and frozen at temperatures between -5 to -60 ^{o}C. The maximal inactivation was observed when the enzyme was stored at -20 ^{o}C, as suggested by the dramatic loss of enzymatic activity. Repeated freezing and thawing did not affect the rate of inactivation. No inactivation was observed in the absence of freezing. The degree of inactivation was directly dependent on Tris-HCl buffer concentrations between 2 mM to 200 mM, but was independent of enzyme concentrations in storage. Under anaerobic conditions, a protective effect against freezing inactivation was observed in the presence of substrates, such as L-leucine and L-arginine. However, there was no protection when the enzyme was reduced anaerobically with dithionite. Glycerol and dimethyl sulfoxide also protected the enzyme. This was explained with the hypothesis that glycerol and methylsulfoxide influenced the ice structure in a way that prevented the formation of the inactive form of the enzyme. Spectroscopic data from protein fluorescence showed that the inactive enzyme

maintained its native conformation. UV-visible absorbance spectra of the enzyme-bound FAD cofactor showed the inactivation of the enzyme was associated with 7 nm and 12 nm hypsochromic shifts of the 462 nm and 390 nm peaks, respectively. Photoreduction in the presence of EDTA was used to examine the reduction properties of the FAD in the inactive enzyme. The rate of the reduction was 10-fold lower than that of active enzyme and only 50% of flavin semiquinone could be formed. Based on spectroscopic and photoreduction data, the authors concluded that the microenvironment of the flavin in the inactive form of the enzyme was significantly affected. The reactivation process of LAAO was also investigated. The enzyme was fully reactivated at pH 5 by increasing the temperature. Adjustment of the pH to 5 or an increase in temperature alone could not reactivate the enzyme; both were required for a full reactivation of LAAO. When the UV-visible absorbance of FAD was monitored during the reactivation process, the absorbance spectrum eventually became similar to that of the native enzyme, which further supported the conclusion that the inactivation of the enzyme was associated with changes in the flavin microenvironment. The temperature-dependence of reactivation yielded a $\Delta H^{\ddagger}$ of 36 to 41 kcal mol^{-1} (151 to 171 kJ mol^{-1}). These large $\Delta H^{\ddagger}$ values from temperature reactivation were in conflict with spectroscopic data, which showed no large protein conformational change. The authors explained the effect of temperature on freezing inactivation using the following model:

$$E_{act} \rightleftharpoons E^* \xrightarrow{k} E_{in}$$

where E_{act} and E_{in} represent the active and inactive forms of enzyme, E* represents a metastable form of enzyme that is in equilibrium with E_{act}, and k represents the rate constant for the conversion from E* to E_{in}. E* was produced as a result of ice formation, therefore $E_{act} \rightleftharpoons E^*$ had large $\Delta H^{\ddagger}$. The rate of inactivation, k, was governed by a small $\Delta H^{\ddagger}$ if the

conversion from $E^* \longrightarrow E_{in}$ involved only a few breakages of chemical interactions. For these reasons, the maximal inactivation was observed at the midpoint temperature, i. e. -20 ^oC, due to high concentration of E* and the rate of inactivation was still significant. When the enzyme was stored at -2 ^oC, no significant inactivation was observed because there was not enough E*. At -60 ^oC, most of the enzyme was in the E* form and *k* had become so low that the inactivation was not detectable anymore.

The freezing-inactivation study by Curti et *al.* was done early before the pH shift in storage buffers was investigated extensively. However, the authors also tested the effect of different buffers on the inactivation of LAAO during freezing. Lower inactivation rates were observed when sodium or potassium phosphate was used as buffers instead of Tris. The authors proposed that phosphates protected the enzyme from freezing inactivation.

5. Hysteretic Behavior in Enzymes

5.1. General description of hysteretic behavior in enzymes. The term "hysteresis" came from physics and was defined as a slow response that was exhibited by a body in reacting to outside forces. Hysteretic behavior of enzyme, on the other hand, was defined as a slow response to a change in ligand concentrations (*96*). Such behavior was observed most often in regulatory enzymes as kinetic cooperativity; however, there have been reports of hysteretic enzymes found in other classes, such as ovoperoxidase (*97*) and leucocyte proteinase (*98*).

In the 1970s, extensive studies on hysteretic enzymes were carried out by Frieden (*96, 99*). In his studies, two mechanisms were believed to be responsible for the slow response in enzymes: 1) isomerization processes and 2) displacement of a tightly bound ligand by another with a different effect on activity. Isomerization processes usually involved conformational

changes in the enzyme that were slow relative to the rate of the reaction (*96*). Generally, the rates of conformational change in enzymes are so fast as compared to the rate of the catalytic reaction that they are difficult to detect. However, it is possible to have multiple local conformational changes that occur one after another causing a delay in enzymatic activity. Displacement reactions involved the dissociation of the ligand from the enzyme (*96*). In the presence of two different ligands, i.e., enzyme inhibitor and substrate, the activity of the enzyme increased upon the displacement of the inhibitor by the substrate at a rate that depended on how rapidly the inhibitor is released from the enzyme surface. Therefore, the longer the inhibitor bound, the longer the lag in enzymatic activity.

5.2. Analysis of hysteretic behavior. The analysis of hysteric behavior in enzymes is fairly complicated because the rate of product accumulation or substrate depletion in activity assays occurs following single exponential function (*99*). Frieden approached the problem by introducing an equation that describes a burst or lag in product appearance (or substrate consumption), which is followed by a linear accumulation of product:

$$v_t = v_\mathrm{f} + (v_\mathrm{i} - v_\mathrm{f})\mathrm{e}^{-kt} \qquad (1)$$

where v_t is the velocity as a function of time, v_f is the velocity after the burst or lag, v_i is the initial velocity, and k the observed rate for the transition of one form to another form of enzyme. This equation is applicable to hysteretic mechanisms with the assumption that the kinetic measurements are performed in the absence of substrate depletion or product accumulation. However, such assumption may affect the evaluation of k if k is dependent on substrate or enzyme concentrations.

Frieden's study on hysteretic behavior of the enzymes extended to detailed analysis of different enzyme systems. The more complicated the system, the more difficult the analysis.

Therefore, it should be noted that the summary provided herein only included a portion of the hysteresis theory that is applicable for the purpose of the study presented in this thesis. For a more detailed treatment of this topic, the reader is referred to Ref. (*96, 99*).

6. Goals

The oxidation of choline to glycine betaine is catalyzed by choline oxidase through two sequential flavin-linked hydride transfers with molecular oxygen as the final electron acceptor and betaine aldehyde as the intermediate. The study of this enzyme is of importance for medical and biotechnological applications because the product of its catalytic reaction, glycine betaine, is a compatible solute that is accumulated in many pathogenic bacteria (*100-109*), and transgenic plants (*12, 16, 18, 20, 110, 111*) in response to adverse hyperosmotic environment (*13, 14*). Therefore, the study of choline oxidase has the potential for the development of therapeutic agents that target the biosynthesis of glycine betaine in human pathogenic bacteria, and for the engineering of drought and salt stress resistance in beneficial bacteria and crop plants. For this reason, the aim of the research presented herein is to investigate the mechanism of enzyme inactivation through mechanism-based inhibition and storage conditions.

To understand the active site structure of choline oxidase is extremely important for the mechanistic study of an enzyme. In this regard, chemical modification of choline oxidase is an effective tool for identifying the active site residues that are essential to catalysis. Active site modifications require the use of irreversible mechanism-based inhibitors as labels. Consequently, the first step in this study was to search for an irreversible inhibitor for choline oxidase using compounds with understood inhibitory mechanisms. Phenylhydrazine and tetranitromethane were found to inactivate choline oxidase to significant extends, so the kinetics

of inactivation and the structural properties of the inactive enzyme were carefully studied in this research.

During the procedure employed to purify choline oxidase, choline oxidase showed a significant hysteretic behavior after storing at pH 6 and -20 ^{o}C (Fan Fan, Prashanthi Menon, Jane V. Hoang, and Giovanni Gadda; unpublished observations), a step used to fully oxidize the enzyme-bound flavin (*30*), suggesting that the enzyme might be affected by freezing at low pH. Since such freezing effects have rarely been reported for enzyme in storage, investigation on the effect of storage temperature and pH on the biophysical and catalytic properties of choline oxidase can give insights into such phenomenon. In addition, storing purified enzymes in frozen solution was almost always employed in our laboratory. Therefore, to understand the inhibitory mechanism of choline oxidase by freezing can help choosing the appropriate conditions for enzymes in storage.

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Chapter II

MATERIAL AND METHODS

1. Materials and Enzyme Preparations

Choline chloride was from ICN Pharmaceutical (Aurora, OH). Glycine betaine was from Sigma-Aldrich (St. Louis, MO). 2-Hydazino-N,N,N-trimethyl-2-oxo-ethanaminium chloride was purchased from Acros Organics N.V. (Fair Lawn, NJ). Trans-2-phenylcyclopropylamine, isonicotinic acid, 3-dimetylamino-1-propyne, allyltrimethylammonium chloride, phenylhydrazine, and tetranitromethane were from Aldrich. TPCK-treated trypsin was purchased from Worthington. Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described previously (*1*). Fully oxidized choline oxidase was prepared as described in ref. (*2*), and was stored at -20 ^{o}C in either 20 mM Tris-Cl, pH 8, or 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6. The enzyme was rapidly thawed on ice prior of each experiment. When needed, buffers were changed either by gel filtration through disposable PD-10 desalting columns (Amersham Pharmacia Biotech, Piscataway, NJ) or dialysis for 19 h at 4 ^{o}C. The concentration of choline oxidase was determined spectrophotometrically at 454 nm using the previously reported ε_{454} value of 11.4 $mM^{-1}cm^{-1}$ (*3*). All kinetic parameters determined were expressed per active site oxidized flavin content (*3*). All other reagents were of the highest purity commercially available.

2. Instruments

UV-visible absorbance spectra were recorded using an Agilent Technologies diode-array spectrophotometer model HP 8453 equipped with a thermostated water bath. Tryptic maps were

obtained using a high performance liquid chromatograph (HPLC) model LC-10AT from Shimadzu. Fluorescence emission spectra were recorded with a Shimadzu Spectrofluorometer model RF-5301 PC thermostated at 15 ^{o}C. Circular dichroism spectra were acquired using a Jasco J-810 spectropolarimeter at 5 ^{o}C. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode (HansaTech Oxy-32) thermostated at 25 ^{o}C.

3. Enzyme Assay

Enzyme activity was measured with the method of the initial rates (*4*) in air-saturated 50 mM potassium phosphate at pH 7 by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 25 ^{o}C. The reactions were started by the addition of choline oxidase to a 1 ml reaction mixture. Unless otherwise stated, the final concentration of enzyme in the reaction mixture was in 0.1 to 0.5 μM range, and the concentration of choline was between 0.05 and 10 mM.

Chemical modification was carried out by incubating the enzyme in 20 mM Tris-Cl at pH 8 and 20 ^{o}C in the presence of ligand. The concentration of ligand in the incubation was in a range from 0.5 and 50 mM. At different times, aliquots were withdrawn and assayed polarographically for enzymatic activity with choline as substrate.

For freezing inactivation study, rates of oxygen consumptions of the enzyme species showing slow onsets of steady state during turnover were determined from the linear portions of the traces, i.e., after steady state was achieved. The pH effects on the fast rate of reactivation of choline oxidase (k_c) were determined by measuring the activity of the enzyme species with

hysteretic behavior in 50 mM [Tris (hydroxymethyl) aminomethane] with the pH in a range from 6.5 to 9.6 at 25 ^{o}C. The temperature effects on the slow rate of activation of choline oxidase (k_{act}) were determined by incubating the inactive enzyme in the range from 20 to 39 ^{o}C at pH 6, and the activity was measured in 50 mM morpholinoethansulfonic acid, at pH 6 and 25 ^{o}C.

4. Tryptic Digestion

To identify the modified site by mechanism-based inhibitor that resulted in enzyme inactivation, the enzyme was incubated with the inhibitors in the presence and absence of 140 mM glycine betaine in 20 mM Tris-Cl at pH 8 and 20 ^{o}C. Enzyme activity was monitored over time until ~90% of the enzyme became inactivated. Gel filtration was used to remove the excess reagents. The inactive enzyme was denatured with 10% (w/v) trichloroacetic acid and incubated on ice for 30 min before centrifugation at 12000*g* for 20 min. The pellets were washed with 10% trichloroacetic acid followed by an ice-cold acetone/HCl (39:1) mixture, resuspended in 0.1 mL of 8 M urea, 4 mM $CaCl_2$, and 0.4 M ammonium bicarbonate, pH 8, and allowed to stand for 1 h at 37 ^{o}C. The solutions were then diluted with 0.3 mL of water before the addition of trypsin to a final concentration of 3% (w/w, trypsin/choline oxidase). After 4 h incubation at 37 ^{o}C, a second aliquot of trypsin (1% w/w final concentration) was added, and the mixture was allowed to react for a further 15 h at 37 ^{o}C. The digestion was stopped with trifluoroacetic acid (1% final concentration). Purification of peptides was carried out by HPLC using a Shimadzu instrument equipped with a model SPD-M10A photodiode array detector and a μBondapak C18 (15 × 4.6 mm) reverse-phase column at a flow rate of 1 mL min^{-1}. Eluent A was 0.05% aqueous trifluoroacetic acid, and eluent B was 0.04% trifluoroacetic acid in acetonitrile. The chromatography was carried out with a linear gradient from 5% to 50% eluent B over 90 min.

Peptides were collected manually. For the identification of the modified peptide, MALDI-TOF mass spectrometry was used in both the positive and negative ion modes with 50:50 methanol/acetonitrile matrix.

5. Spectral Studies

In freezing inactivation study, absorbance, fluorescence, and circular dichroic spectra were determined in 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6, at 15 ^{o}C. The concentration of enzyme in flavin content was: 11 μM for the UV-visible absorbance spectra, 3 μM for the protein fluorescence emission spectra (with λ_{ex} at 285 nm), 11 μM for the flavin fluorescence emission (with λ_{ex} at ~455 nm), and 2.5 μM, 50 μM, and 168 μM for the CD spectra of the protein in the far-UV, near-UV, and visible regions of the spectra, respectively.

6. Data Analysis

Data were fit with KaleidaGraph software (Synergy Software, Reading, PA) and Enzfitter software (Biosoft, Cambridge, UK). The steady state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation for one substrate. Time course of inactivation of choline oxidase was fit with eq (1), where A represents the residual activity at a given time (t), A_0 is the initial activity, and k_{obs} is the observed rate of the inactivation. The rates of inactivation of the enzyme were fit to eq (2), where k_{inact} is the first-order rate constant for the conversion of the reversibly formed enzyme-inhibitor complex to irreversibly inactivated enzyme, I is the concentration of the mechanism-based inhibitor, and K_I is the dissociation constant of the mechanism-based inhibitor from the enzyme.

$$A = A_0 e^{-k_{obs} t} \qquad (1)$$

$$k_{\text{obs}} = \frac{k_{\text{inact}}[\text{I}]}{[\text{I}] + K_{\text{I}}} \qquad (2)$$

In freezing inactivation study, the first-order rate constants for the fast and pH-dependent activation of the inactive form of choline oxidase (k_c) were determined by fitting the time-courses of oxygen consumption to eq 3, which was modified from Frieden (*5*), where v_f represents the observed rate of reaction after reaching steady state, and $[O_2]_o$ is the initial concentration of oxygen. The pH dependence of k_c was determined by using eq 4, which describes a curve with a slope of +1 and a plateau region at high pH where C represents the pH-independent value. The apparent rates of activation of the enzyme at any given temperature were determined by fitting the observed rates of oxygen consumption at any given incubation time with eq 5 that describes a single exponential process. Here, ${}^{\text{app}}k_{\text{act}}$ is the apparent first-order rate constant for the temperature-dependent slow activation of the inactive form of choline oxidase, t is time, A_t is the value of enzymatic activity, A_o is the amplitude of the total change, and A_∞ is the enzymatic activity at infinite time. The temperature dependence of k_{act} was determined by using the Eyring's equation (eq 6), where k_{act} is the first-order rate constant for enzyme activation, k_B and h are the Boltzmann and Planck constants, T is the absolute temperature in Kelvin, R is the gas constant with a value of 8.315 J mol^{-1} K^{-1}, and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively.

$$\Delta[\text{O}_2]_{\text{t}} = v_{\text{f}} t - v_{\text{f}} \frac{1 - e^{-\text{k}_{\text{c}} t}}{k_{\text{c}}} + [\text{O}_2]_{\text{o}} \qquad (3)$$

$$\log k_{\text{c}} = \log\left(\frac{C}{1 + \frac{10^{-\text{pH}}}{10^{-\text{p}K\text{a}}}}\right) \qquad (4)$$

$$A_t = A_\infty - A_o \text{e}^{-t\,{}^{app}k_{\text{act}}} \qquad (5)$$

$$\ln\left(\frac{k_{\text{act}}}{\text{T}}\right) = \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^{\ddagger}}{\text{R}} - \frac{\Delta H^{\ddagger}}{\text{RT}} \qquad (6)$$

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Chapter III

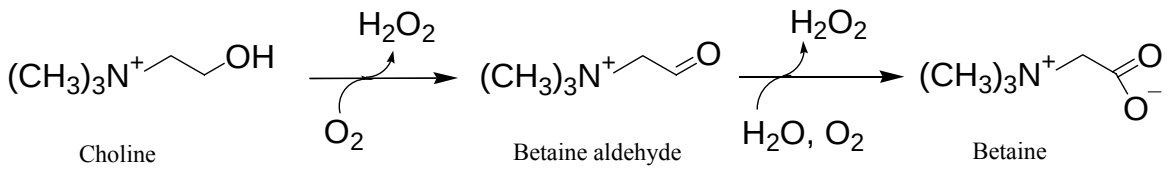
CHEMICAL MODIFICATION OF CHOLINE OXIDASE: THE SEARCH FOR THE INHIBITOR

Abstract

Choline oxidase is a flavin-dependent enzyme that catalyzes the oxidation of choline to glycine-betaine, with oxygen as electron acceptor. In the absence of crystallographic data, chemical modification was employed in order to identify essential catalytic residues in the active site of choline oxidase. Because mechanism-based inhibitors are needed for the modification of enzyme, seven reagents were tested in this study as inhibitors for choline oxidase: 2-hydrazino-*N,N,N*-trimethyl-2-oxopethanaminium, allyltrimethylammonium, isonicotinic acid hydrazide, 3-dimethylamino-1-propyne, *trans*-2-phenylcyclopropylamine, phenylhydrazine, and tetranitromethane. The results showed significant inactivation of the enzyme with tetranitromethane and phenylhydrazine at concentration ranging from 0.5 to 5.0 mM and 0.1 to 2.0 mM, respectively. The observed inactivation rates at these concentrations ranged from 0.1 to 0.4 h^{-1} with tetranitromethane and from 0.5 to 4.4 h^{-1} with phenylhydrazine. Enzyme inactivation was also observed with *trans*-2-phenylcyclopropylamine in the concentration range from 10 to 50 mM to yield a k_{obs} value from 0.2 to 0.3 h^{-1}, respectively. The results in this study helped identifying three possible inhibitors that might be used for the modification of choline oxidase.

Introduction

Choline oxidase (CHO; E.C. 1.1.3.17) from *Arthrobacter globiformis* catalyzes the oxidation of choline to glycine betaine through two flavin-linked sequential hydride transfers with betaine aldehyde as the intermediate and molecular oxygen as the final electron acceptor (Scheme 3.1).



Scheme 3.1. Reaction catalyzed by choline oxidase.

The enzyme is a homodimer of 120 kDa, with each monomer containing covalently bound FAD (*1*). The study of choline oxidase was considered of importance to both medical and biotechnological applications because the product of its catalytic reaction, glycine betaine, was found to accumulate in the cytoplasm of cells to prevent dehydration and plasmolysis in adverse hyperosmotic environments (*2, 3*) in pathogenic bacteria (*4-13*) and transgenic plants (*14-19*). Consequently, the study of choline oxidase has potential for the development of therapeutic agents targeted at the inhibition of glycine betaine biosynthesis and for the engineering of drought resistance in economically relevant crops.

In earlier work, we have reported the cloning and expression of the gene coding for choline oxidase from *A. globiformis* strain ATCC 8010 in *E. coli* (*1*). The chemical mechanism for the oxidation of choline catalyzed by choline oxidase has been recently elucidated using kinetic isotope effects, and is consistent with the quantum mechanical transfer of a hydride from the substrate α-carbon to the enzyme-bound flavin cofactor occurring from an activated alkoxide form of choline (*20, 21*). Such an alkoxide species has been proposed to form by the action of an

active site base with pK_a of ~7.5 (*22-24*), and to be electrostatically stabilized in the enzyme active site by the imidazolium side chain of His_{466} (*25, 26*).

Despite the wealth of information that has previously been obtained on choline oxidase using kinetic and mechanistic studies, the lack of 3-dimensional structure hindered the understanding of the catalytic reaction at structural level. In the absence of crystallographic data, chemical modification is a powerful technique that can be used to identify the essential residues in the active site of enzyme. This technique involves experiment with mechanism-based inhibitors of the enzyme. If the inhibitor reacts with side chains of an amino acid and lead to enzyme inactivation, then it is very likely that this amino acid plays important roles in enzyme function, such as participating directly in catalytic process, participating in binding interaction with a substrate, or stabilizing the enzyme conformation that is required for catalysis. In this chapter, several reagents were employed for the chemical modification studies of choline oxidase, in which only the highly reactive and selective reagents will be chosen for more detailed study.

Material and Methods

Materials and enzyme preparations. Choline chloride was from ICN Pharmaceutical (Aurora, OH). Glycine betaine was from Sigma-Aldrich (St. Louis, MO). 2-hydazino-N,N,N-trimethyl-2-oxo-ethanaminium chloride was purchased from Acros Organics N.V. (Fair Lawn, NJ). Trans-2-phenylcyclopropylamine, isonicotinic acid, 3-dimetylamino-1-propyne, allyltrimethylammonium chloride, phenylhydrazine, and tetranitromethane were from Aldrich. TPCK-treated trypsin was purchased from Worthington (Lakewood, NJ). Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described previously (*1*). Fully oxidized choline oxidase was prepared as described in ref. (*24*), and was stored at -20 ^{o}C in either 20 mM Tris-Cl, pH 8, or 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6. The enzyme was rapidly thawed on ice prior of each experiment. The concentration of choline oxidase was determined spectrophotometrically at 454 nm using the previously reported ε_{454} value of 11.4 $mM^{-1}cm^{-1}$ (*23*). All kinetic parameters determined were expressed per active site oxidized flavin content (*23*). All other reagents were of the highest purity commercially available.

Instruments. Inactivation of enzyme was carried out by incubating the enzyme with the inhibitor in Eppendorf Mastercycler model 5333 thermostated at 20 ^{o}C. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode (HansaTech Oxy-32) thermostated at 25 ^{o}C.

Enzyme assays. The enzyme was incubated in 20 mM Tris-Cl at pH 8 and 20 ^{o}C in the presence of chosen reagent. At different times, aliquots were withdrawn and assayed polarographically for enzymatic activity. Enzyme activity was measured with the method of the initial rates (*27*) in air-saturated 50 mM potassium phosphate at pH 7 by monitoring the rate of

oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 25 ^{o}C. The reactions were started by the addition of choline oxidase to a 1 ml reaction mixture. Unless otherwise stated, the final concentration of enzyme in the reaction mixture was in the 0.1 to 0.5 μM range, and the concentration of choline was 10 mM.

Data analysis. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA). The steady state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation for one substrate. Time course of inactivation of choline oxidase was fit with eq (1), where A represents the residual activity at a given time (t), A_0 is the initial activity, and k_{obs} is the observed rate of the inactivation.

$$A = A_0 e^{-k_{obs} t} \qquad (1)$$

Results

In order to ensure that enzyme inactivation was only due to the reaction of choline oxidase with the reagent being tested, a control experiment was carried out in which the enzyme was incubated in 20 mM Tris-Cl, pH 8, at 20 ^{o}C. As show in Figure 3.1, the residual activity of choline oxidase remained constant up to two hours of incubation; therefore, all inactivation experiments were done under these conditions.

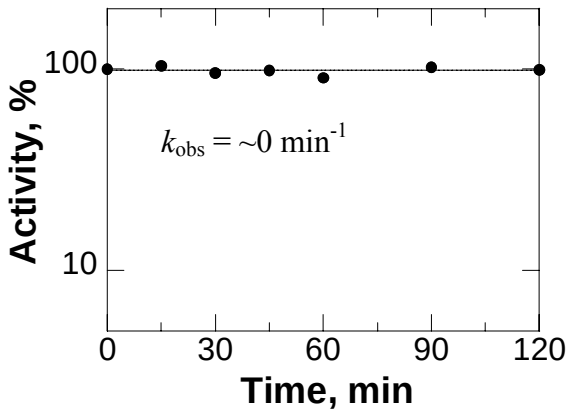
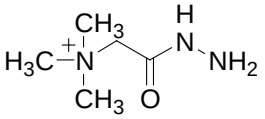
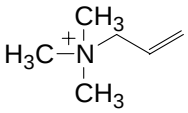
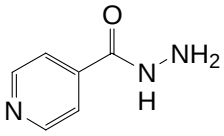
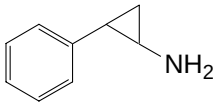
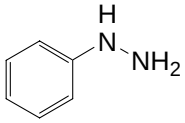
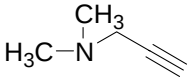
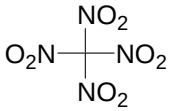


Figure 3.1. Stability of choline oxidase in 20 mM Tris-Cl, pH 8, at 20 ^{o}C. Enzyme activity was measured by monitoring the rate of oxygen consumption in air-saturated 50 mM potassium phosphate at pH 7 and 25 ^{o}C. The data were fit with Eq. (1).

Seven reagents were chosen to test as inhibitors of choline oxidase in 20 mM Tris-Cl, pH 8, at 20 ^{o}C (Table 3.1). The choice of reagents was based on structural similarity of the reagents with the substrates or product of the enzyme or the previously studied inhibitory mechanism of the reagents. As shown in Table 3.1, there was no enzyme inactivation observed with 2-hydrazino-N,N,N-trimethyl-2-oxopethanaminium, allyltrimethylammonium, isonicotinic acid hydrazide, and 3-dimethylamino-1-propyne. In contrast, significant enzyme inactivation was observed with phenylhydrazine and tetranitromethane. *Trans*-2-phenylcyclopropylamine also inactivated choline oxidase but at an observed rate that was ~200-fold less than that of phenylhydrazine and 40-fold slower than that of tetranitromethane.

Table 3.1. List of reagents tested as mechanism-based inhibitors for choline oxidase.[a]

Reagent name	Structure	Concentrations, mM	$k_{obs,}$[b] h^{-1}
2-hydrazino-N,N,N-trimethyl-2-oxo-ethanaminium		20-50	0
allyltrimethylammonium		20-50	0
isonicotinic acid hydrazide		20-50	0
trans-2-phenylcyclopropylamine		10-50	0.2-0.3
phenylhydrazine		0.1-2.0	0.5-4.4
3-dimethylamino-1-propyne		10-50	0
tetranitromethane		0.5-5.0	0.1-0.4

[a] Data were obtained by incubating the enzyme with the reagent in 20 mM Tris-Cl, pH 8, at 20 °C. Activity was monitored over time with choline as substrate in 50 mM potassium phosphate, pH 7, at 25 °C.
[b] Observed rate of enzyme inactivation, which was determined by fitting the residual activity at a given time *t* to the single exponential decay equation (Eq. 1).

Discussion

The flavoprotein choline oxidase from *Arthrobacter globiformis* catalyzes the two steps oxidation of a primary alcohol substrate to a carboxylate product with an aldehyde intermediate. In the absence of crystallographic data, chemical modification was employed in order to identify essential catalytic residues in the active site of choline oxidase. The study presented herein helped identifying mechanism-based inhibitors that may be used for enzyme modification. Out of the seven reagents tested (Table 3.1), the enzyme showed significant inactivation rates only with tetranitromethane, phenylhydrazine, and *trans*-2-phenylcyclopropylamine. Tetranitromethane (TNM) has been used for chemical modification of amino acid residues in peptides, polymers, and proteins since 1930 (*28-30*). This reagent is well known for its high specificity for the nitration of tyrosyl residues with distinct visible absorbance that can be quantitated using spectrophotometry (*30*). Phenylhydrazine and *trans*-2-phenylcyclopropylamine has been used in modification of flavoenzymes such as monoamine oxidase (*31, 32*) and trimethylamine dehydrogenase (*33, 34*). Enzyme inactivation with these two reagents usually involves the generation of a carbon-centered radical or anion at the enzyme active site, resulting in the formation of flavin C4(a) adduct. In this study, significant inactivation rates were observed for choline oxidase with all three reagents; however, more specific experiments must be performed in order to distinguish the amino acid that has been modified. Detailed studies on the modification of choline oxidase with phenylhydrazine and tetranitromethane are illustrated in Chapter 4 and Chapter 5, respectively.

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Chapter IV

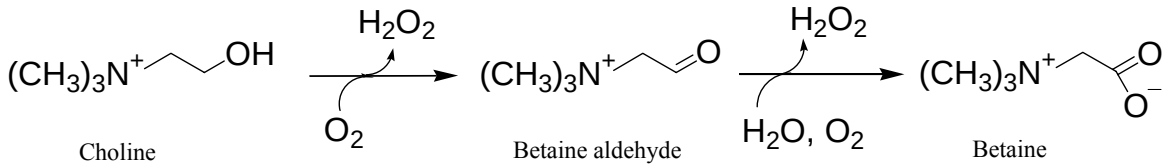
CHEMICAL MODIFICATION OF CHOLINE OXIDASE WITH PHENYLHYDRAZINE

Abstract

The four electron-oxidation of choline to glycine betaine was catalyzed choline oxidase through two flavin-dependent hydride-transfer steps within the same active site. When choline oxidase was incubated with phenylhydrazine in 20 mM Tris-Cl at pH 8 and 20 ^{o}C, there was an irreversible loss of enzymatic activity. The inactivation of choline oxidase by phenylhydrazine was both time and concentration dependent with a second-order rate constant of 0.04 mM^{-1} min^{-1}. The enzyme was fully protected from inactivation when there was a ligand in the enzyme active site, indicating the inactivation by phenylhydrazine is active-site directed. Phenylhydrazine could be use as a substrate of choline oxidase, but substrate-inhibition mechanism was observed at concentration ≥20 mM. The inactivation of the enzyme by phenylhydrazine associated with the bleaching of the 454 nm peak and an increase in absorbance at 370 nm, suggesting the formation of flavin semiquinone. However, the percentage of remaining residual activity was 3 times lower than the remaining oxidized enzyme; therefore, the generation of flavin semiquinone is not fully responsible for enzyme inactivation. The tryptic maps of protected and unprotected enzyme samples were compared, but no specific modification site was identified due to nonselective reactivity of phenylhydrazine.

Introduction

Choline oxidase (CHO; E.C. 1.1.3.17) from *Arthrobacter globiformis* was first purified by Ikuta *et. al.* in 1977 (*1*). The enzyme is a homodimer with a mass of 120 kDa that catalyzes the oxidation of choline to glycine betaine through two flavin-linked sequential hydride transfers with betaine aldehyde as the intermediate and molecular oxygen as the final electron acceptor (Scheme 4.1).



Scheme 4.1. Reaction catalyzed by choline oxidase.

The study of choline oxidase is of interest to the understanding of glycine betaine biosynthesis in pathogenic bacteria (*2-11*) and transgenic plants (*12-17*) as a response dehydration and salt stress in adverse hyperosmotic environments (*18, 19*). The detail biosynthesis mechanism of glycine betaine can be used in the research for therapeutic agents that target the inhibition of glycine betaine biosynthesis and for the engineering of drought resistance in economically relevant crops.

In the past decade, the biotechnological and medical applications of choline oxidase have been studied extensively, but there was no reported study on the catalytic mechanism of the enzyme. Until just recently, we have reported the cloning and expression of the gene coding for choline oxidase from *A. globiformis* strain ATCC 8010 in *E. coli* (*20*). With the excess availability of the enzyme, a number of kinetics and mechanistic studies have been carried out in our laboratory. The chemical mechanism for the oxidation of choline was understood based on data from kinetic isotope effects, which suggested the quantum mechanical transfer of a hydride from the substrate α-carbon to the enzyme-bound flavin cofactor occurring from an activated

alkoxide form of choline (*21, 22*). The pH and kinetic isotope effects on the kinetic parameters k_{cat} and k_{cat}/K_m with choline as substrate gave evidence to the participation of active site base with pK_a of ~7.5 in catalysis (*23, 24*). The roles of His466 in the active site of choline oxidase were also established by substituting it with an alanine or an asparatate (*25, 26*). Based on spectroscopic and kinetic properties of H466A and H466D mutants, His466 is believed to be responsible for the stabilization of the negative charges on the alkoxide species of choline and on the N(1)-C(2)=O region of the flavin and contributed into the polarity of the active site of choline oxidase.

Even though we have gained insights into the catalytic mechanism of choline oxidase with kinetic and mechanistic techniques, the understanding of detailed catalytic strategy of this enzyme is still limited at structural level. In the absence of X-ray crystallographic data, chemical modification with irreversible inhibitors can be used to identify essential residue in the active site of the enzyme. In this study, phenylhydrazine was used to inactivate the enzyme. The kinetic and mechanistic data from the inactivation were determined using spectrophotometric and chromatographic techniques. Identification of the modification site that responsible for the inactivation of the enzyme were performed using tryptic digestion, HPLC, and mass spectrometry.

Experimental Procedures

Materials and enzyme preparations. Choline chloride was from ICN Pharmaceutical (Aurora, OH). Glycine betaine and phenylhydrazine were from Sigma-Aldrich (St. Louis, MO). TPCK-treated trypsin was purchased from Worthington (Lakewood, NJ). Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described previously (*20*). Fully oxidized choline oxidase was prepared as described in ref. (*27*), and was stored at -20 ^{o}C in either 20 mM Tris-Cl, pH 8, or 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6. The enzyme was rapidly thawed on ice prior of each experiment. The concentration of choline oxidase was determined spectrophotometrically at 454 nm using the previously reported ε_{454} value of 11.4 $mM^{-1}cm^{-1}$ (*24*). All kinetic parameters determined were expressed per active site oxidized flavin content (*24*). All other reagents were of the highest purity commercially available.

Instruments. Inactivation of enzyme was carried out by incubating the enzyme with the inhibitor in Eppendorf Mastercycler model 5333 thermostated at 20 ^{o}C. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode (HansaTech Oxy-32) thermostated at 25 ^{o}C. UV-visible absorbance spectra were recorded on a Hewlett-Packard 8453A diode array spectrophotometer. Purification of peptides was carried out by HPLC using a Shimadzu instrument equipped with a model SPD-M10A photodiode array detector and a μBondapak C18 (15 × 4.6 mm) reverse-phase column.

Enzyme assays. Enzyme activity was measured with the method of the initial rates (*28*) in air-saturated 50 mM potassium phosphate at pH 7 by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 25 ^{o}C. The reactions were started by the addition of choline

oxidase to a 1 ml reaction mixture. Unless otherwise stated, the final concentration of enzyme in the reaction mixture was in 0.1 to 0.5 μM range, and the concentration of choline was 10 mM. The enzyme was inactivated by incubating in 20 mM Tris-Cl at pH 8 and 20 ^{o}C in the presence of phenylhydrazine. At different times, aliquots were withdrawn and assayed polarographically for enzymatic activity. Stock solution of phenylhydrazine was prepared just prior to use.

Tryptic Digestion. To identify the modified site by mechanism-based inhibitor that resulted in enzyme inactivation, the enzyme was incubated with phenylhydrazine in 20 mM Tris-Cl at pH 8 and 20 ^{o}C in the presence and absence of 140 mM glycine betaine. Enzyme activity was monitored over time until ~90% of the enzyme became inactivated. Gel filtration was used to remove the excess reagents. The inactive enzyme was denatured with 10% (w/v) trichloroacetic acid and incubated on ice for 30 min before centrifugation at 12000*g* for 20 min. The pellets were washed with 10% trichloroacetic acid followed by an ice-cold acetone/HCl (39:1) mixture, resuspended in 0.1 mL of 8 M urea, 4 mM $CaCl_2$, and 0.4 M ammonium bicarbonate, pH 8, and allowed to stand for 1 h at 37 ^{o}C. The solutions were then diluted with 0.3 mL of water before the addition of trypsin to a final concentration of 3% (w/w, trypsin/choline oxidase). After 4 h incubation at 37 ^{o}C, a second aliquot of trypsin (1% w/w final concentration) was added, and the mixture was allowed to react for a further 15 h at 37 ^{o}C. The digestion was stopped with trifluoroacetic acid (1% final concentration). Purification of peptides was carried out by HPLC using a Shimadzu instrument equipped with a model SPD-M10A photodiode array detector and a μBondapak C18 (15 × 4.6 mm) reverse-phase column at a flow rate of 1 mL min^{-1}. Eluent A was 0.05% aqueous trifluoroacetic acid, and eluent B was 0.04% trifluoroacetic acid in acetonitrile. The chromatography was carried out with a linear gradient from 5% to 50% eluent B over 90 min. Peptide fragments were collected manually. For

the identification of the modified peptide, MALDI-TOF mass spectrometry was used in both the positive and negative ion mode with 50:50 methanol/acetonitrile matrix.

Data Analysis. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA). The steady state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation for one substrate. Time course of inactivation of choline oxidase was fit with eq (1), where A represents the residual activity at a given time (t), A_0 is the initial activity, and k_{obs} is the observed rate of the inactivation. Substrate inhibition data was fit to eq (2) (*29*), where v is the velocity of enzyme activity at any concentration S, V_{max}' is the observed maximal velocity, [S] is the concentration of the substrate, K_m' is the apparent Michaelis-Menten constant, and K_I is the dissociating constant of the inhibitor from the enzyme.

$$A = A_0 e^{-k_{obs} t} \qquad (1)$$

$$v = \frac{V_{max}'[\mathrm{S}]}{K_m' + [\mathrm{S}] + K_I[\mathrm{S}]^2} \qquad (2)$$

Results

Inactivation of Choline Oxidase with Phenylhydrazine. When choline oxidase was incubated with phenylhydrazine in 20 mM Tris-Cl at pH 8 and 20 ^{o}C, a time-dependent loss of enzymatic activity was observed (Figure 4.1 panel A). In the presence of glycine betaine, the enzyme remained ~100% active with 0.5 mM phenylhydrazine, suggesting that the enzyme is protected from inactivation in the presence of its catalytic product. As shown in Figure 4.1 panel B, the inactivation followed second-order kinetics that is dependent on the concentration of phenylhydrazine and yields a k_{inact} value of 0.04 mM^{-1} min^{-1}. After inactivated enzyme was isolated by gel filtration and was incubated in 20 mM Tris-Cl at pH 8 and 20 ^{o}C for three hours, no recovery of activity was observed. This indicated that the inactivation of choline oxidase by phenylhydrazine was irreversible. Phenylhydrazine was also tested as a substrate for choline oxidase in 50 mM potassium phosphate at pH 7 and 25 ^{o}C. Significant activity was detected with phenylhydrazine concentrations in the range from 5 mM to 40 mM. The activity of the enzyme increased with increasing phenylhydrazine concentration up to 20 mM then gradually decreased at concentrations >20 mM, suggesting a substrate inhibition mechanism (*29*). The data were fit with Equation 2 to yield a k_{cat} of 1.1 ± 0.8 s^{-1}, a K_m' of 34.7 ± 27.8 mM, and a K_I of 8.5 ± 7.2 mM. The large error obtained from the fitting may due to not enough data points to define the curve.

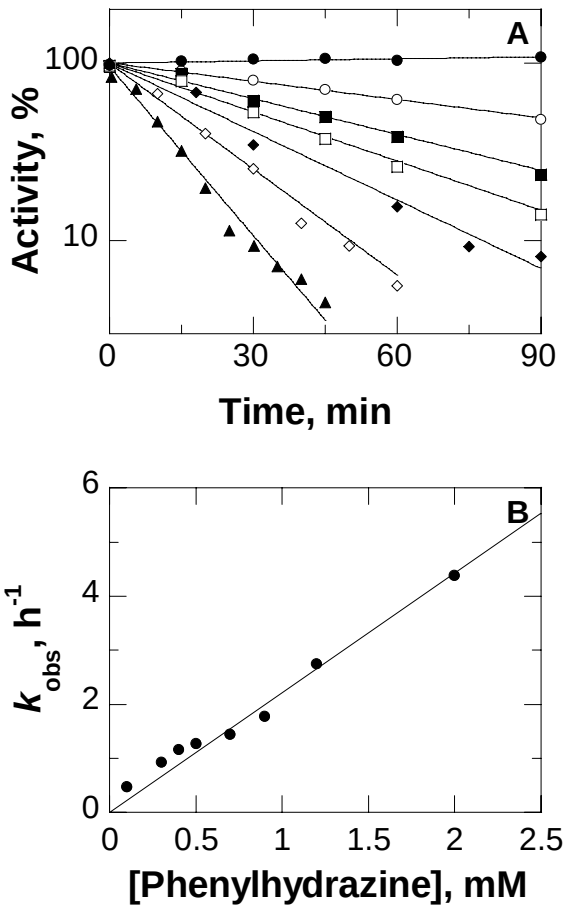


Figure 4.1. Time-dependent inactivation of choline oxidase by phenylhydrazine. Choline oxidase at 0.54 μM was incubated with different concentrations of phenylhydrazine in 20 mM Tris-Cl, pH 8, at 20 ^{o}C. Enzyme activity was monitored overtime in 50 mM potassium phosphate, pH 7, at 25 ^{o}C. (A) Time course inactivation with phenylhydrazine at (●) 0.5 mM in the presence of 1.2 M glycine betaine, (○) 0.1 mM, (■) 0.3 mM, (□) 0.5 mM, (♦) 0.9 mM, (◊) 1.2 mM, and (▲) 2.0 mM. (B) The observed rate of enzyme inactivation as a function of the concentration of phenylhydrazine.

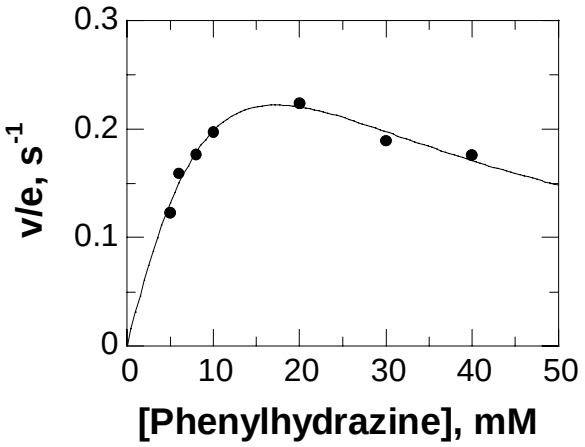


Figure 4.2. Substrate inhibition behavior of choline oxidase with phenylhydrazine as substrate. Enzyme activity was measured in 50 mM potassium phosphate, pH 7, at 25 ^{o}C. The curve is a fit of the data to equation (2).

Properties of Choline Oxidase Modified with Phenylhydrazine. UV-visible absorbance spectrophotometer was used to observe the effect of phenylhydrazine under aerobic conditions on the flavin cofactor of choline oxidase in 20 mM Tris-Cl at pH 8 and 15 ^{o}C. As shown in panel A of Figure 4.3, the inactivation of the enzyme by phenylhydrazine was associated with the bleaching of the 454 nm peak and an increase in absorbance at 370 nm, suggesting the formation of flavin semiquinone. After 2.5 h of incubation, 86% of residual activity was lost and 67% of oxidized flavin was turned into flavin semiquinone (Figure 4.3 panel B). The reduced form of the enzyme-bound flavin could not be obtained with longer incubation time (up to 30 h) or with higher concentrations of phenylhydrazine (up to 2 mM).

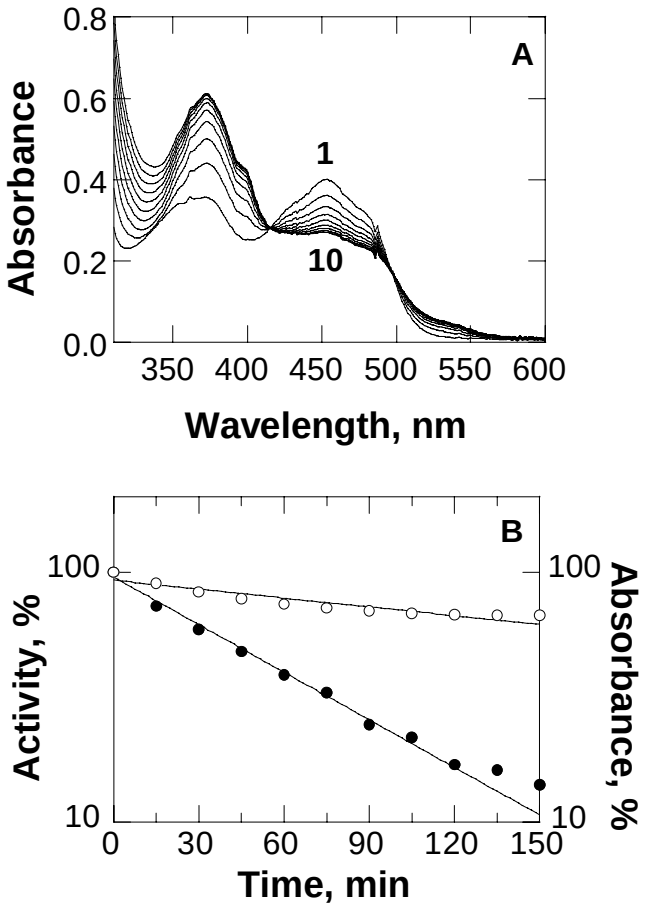


Figure 4.3. UV-visible absorbance spectra of the inactivation of choline oxidase at 35.2 μM with 0.5 mM phenylhydrazine in 20 mM Tris-Cl, pH 8, at 15 ^{o}C. (A) For clarity, only selected UV-visible absorbance spectra were showed; spectra **1** and **10** were recorded at 1 and 150 min of incubation, respectively. (B) Residual activity (●) and absorbance at 455 nm (○); the data were fit with equation (1).

Identification of the Site of Modification of Choline Oxidase by Phenylhydrazine. To identify the site of modification by phenylhydrazine that is responsible for the inactivation of choline oxidase, the enzyme was inactivated to 20% residual activity. Excess phenylhydrazine was removed by gel filtration. The sample was digested with trypsin into peptide fragments. The peptides were separated by reverse-phase HPLC and compared to the peptides fragment of another enzyme sample that was treated with the same concentration of phenylhydrazine but was protected by glycine betaine (Figure 4.4). The two chromatographic maps were similar to each other except for the peptides eluted at 44.3 min, 50.9 min, and 60.7 min, which showed a significant difference in peak intensity. These peptides were collected and identified using mass spectrometry (Table 4.1). The results from mass spectrometry showed that the peptide fragments eluted at the three elution times were found in both protected and unprotected enzyme sample, suggesting no specific modification on the peptides. However, at 50.9 min, the unprotected sample had one extra peak that could not be identified because its mass did not match with any of the possible modified or unmodified tryptic digested peptides.

Table 4.1. Mass spectrometry analysis of modified peptides in choline oxidase.[a]

Elution time, min	44.3		50.9		60.7	
In the presence (+GB) or in the absence (-GB) of glycine betaine	+GB	-GB	+GB	-GB	+GB	-GB
Molecular weight of peptide from mass spectrometry analysis, m/z	1828.8 1921.8 2193.0	1828.6 1921.6 2191.7	1837.8	1838.6 1860.5	3208.0	3207.7

[a] Data obtained with MALDI-TOF mass spectrometry, using both positive and negative ion mode with 50:50 methanol/acetonitrile matrix.

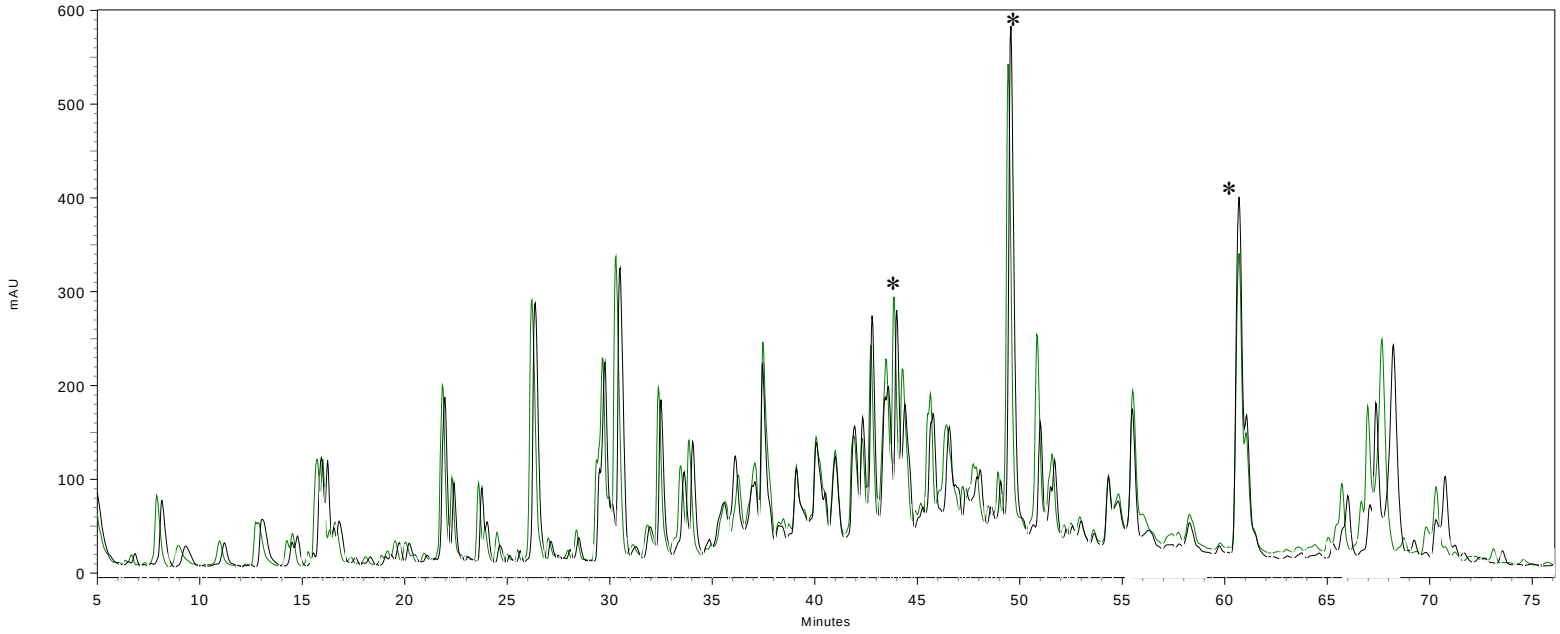


Figure 4.4. Overlaid HPLC chromatograms of tryptic digests of choline oxidase treated with phenylhydrazine in the presence (black line) and absence (green line) of glycine betaine. Peptide elution was monitored at 214 nm with a photodiode array spectrophotometer detector. The peptides eluting at 44.3 min, 50.9 min, and 60.7 min (*) showed a significant difference in peak intensity.

Discussion

The flavoprotein choline oxidase from *Arthrobacter globiformis* catalyzes the two steps oxidation of a primary alcohol substrate to carboxylate product with an aldehyde intermediate. In the absence of crystallographic data, chemical modification was employed in order to identify the essential catalytic residues in the active site of choline oxidase. In this study, phenylhydrazine was chosen for the modification study of choline oxidase because it can inactivate the enzyme at low concentrations. The inhibitory mechanism of enzymes by phenylhydrazine has also been studied by other authors and understood for other flavoenzymes, such as monoamine oxidase (*30*) and trimethylamine dehydrogenase (*31*) Enzyme inactivation by phenylhydrazine usually involves the generation of a carbon-centered radical or anion at the active site (*31, 32*). These species are highly unstable and could react with the cofactors or active site residues in the enzyme.

The results presented herein show that the inactivation of choline oxidase by phenylhydrazine is both time and concentration dependent. The evidence for this conclusion comes from the single exponential decay of enzymatic activity in the presence of phenylhydrazine. The observed rate of inactivation also increased with increasing concentration of phenylhydrazine yielding a second-order rate constant, suggesting a reversible inactivation. However, there was no recovery of activity when the inactive enzyme was isolated and incubated at 20 ^{o}C for 3 h. A likely explanation for these results is that the concentrations of phenylhydrazine used to obtain k_{obs} of inactivation are still bellow K_I value (the dissociation constant of the mechanism-based inhibitor) i. e., larger concentration of phenylhydrazine is required to reach k_{inact}. However, reaching kinact can be difficult to achieve because the

observed rate of inactivation of the enzyme with phenylhydrazine >2.0 mM will be too fast to measure.

The inactivation of choline oxidase is active-site directed, as suggested by the protective effect of glycine betaine on the enzyme. This conclusion is consistent with the fact that phenylhydrazine can be used as the substrate of choline oxidase. These data implied that the inactivation of choline oxidase with phenylhydrazine is mechanism-based; the inhibitor is required be converted into its reactive form for the inactivation to occur. However, more data are needed to support this interpretation because it is predicted that the flavin peak will be bleached reversibly by phenylhydrazine under anaerobic conditions if there is an oxidative process of the inhibitor involved, as previously reported for the case with the flavin-dependent monoamine oxidase (*33*).

The formation of flavin semiquinone upon incubation with phenylhydrazine is not fully responsible for the inactivation of choline oxidase, as suggested by the difference in the remaining enzymatic activity and the oxidized form of the enzyme[3] after 2.5 h incubation with phenylhydrazine. This is consistent with the involvement of one electron transfer of the flavin in the process of enzyme inactivation. To our knowledge, there was no previously reported electron-transfer inhibitory mechanism observed for the case of flavoenzyme with phenylhydrazine as the inhibitor. Instead, phenylhydrazine was well known for inhibition involving the generation of the phenyl radical, whose reactivity is nonselective (*34*). In fact, it is

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[3] Earlier study by our group reported the semiquinone form of choline oxidase is insensitive to both oxygen and organic substrate, as suggested by the lack of oxygen consumption and the lack of change in UV-visible absorbance of the flavin semiquinone in the presence of choline *(24)*. Therefore, if the semiquinone formation is fully responsible for the inactivation of choline oxidase with phenylhydrazine, the remaining enzymatic activity at the end of incubation should be comparable to the amount of oxidized enzyme remained. That is because the oxidized and unmodified-enzyme was the only species that can consume oxygen in the activity assay.

very likely that the inactivation process of choline oxidase with phenylhydrazine involving both one electron-transfer of the flavin and the nonselective reaction of phenyl radicals with group(s) in the active site of the enzyme that participate in catalysis. This hypothesis can also be used to explain the various modification sites found on tryptic map of the inactive enzyme. Nevertheless, more data must be obtained in order to prove this hypothesis.

We concluded that the chemical modification of choline oxidase using phenylhydrazine resulted in nonspecific modification; accounting for the fact of many modifications was found but no modification site that responsible for enzyme inactivation was identified. However, modification with higher concentration of phenylhydrazine might be used to reduce the interaction time of the inhibitor with the enzyme environment, resulting in more selective modification. Future studies will aim to obtain a more selective modification of choline oxidase using phenylhydrazine. The identification of a residue that is of importance to the catalytic mechanism of choline oxidase could be used in future mutagenesis to better understand the catalytic strategy of the enzyme.

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Chapter V

CHEMICAL MODIFICATION OF CHOLINE OXIDASE WITH TETRANITROMETHANE

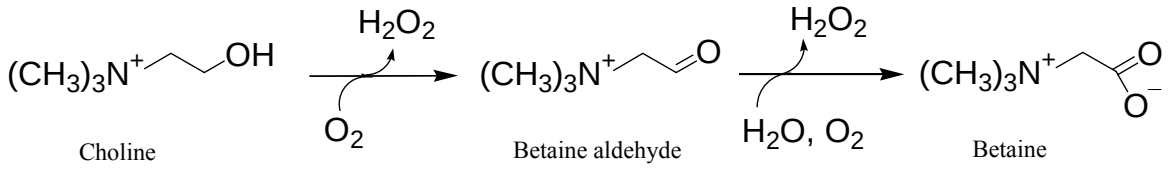
Abstract

Choline oxidase catalyzes the two sequential flavin-linked hydride transfers that convert choline to glycine betaine. In this study, the enzyme was irreversibly inactivated with tetranitromethane in 20 mM Tris-Cl at pH 8 and 20 ^{o}C. The inactivation by tetranitromethane was both time and concentration dependent. The rate of inactivation followed saturation kinetics, yielding a first-order rate constant (k_{inact}) of 0.32 ± 0.01 h^{-1}, and a dissociation constant (K_I) of 0.66 ± 0.04 mM, suggesting the inactivation occurred after tetranitromethane bound to the enzyme. The product of the enzyme catalytic reaction, glycine betaine, protected the enzyme from inactivation, indicating the inactivation is active-site directed. UV-visible absorbance of both protected and unprotected enzyme sample showed the presence of nitrated tyrosines, but the unprotected sample had greater absorbance intensity equivalent to a difference of 5 tyrosines. The tryptic maps of protected and unprotected enzyme sample were compared, but the modifications by tetranitromethane were found on both enzyme samples; therefore, no specific modification site was identified.

Introduction

Tetranitromethane (TNM) has been used in chemical modification of many proteins since 1930 (*1*). In many cases, incubation with this reagent resulted in the irreversible inactivation of the enzyme. The inactivation may be active-site-directed, as shown by the protective effects of competitive inhibitors. Detailed studies of on the protein modification by tetranitromethane have suggested that the reaction of TNM involves the nitration of tyrosyl residues (*2, 3*). The reaction of tetranitromethane with tyrosyl residue involve the formation of a charge-transfer complex with the phenol group of the tyrosine, followed by the generation of the phenoxide and nitrite radicals due to electron transfer. The nitrite radicals reacts with the phenoxide and results in the formation of the nitrotyrosine and the release of a nitroformate anion. Additional reactions are possible due to the high reactivity of phenolate radicals with each other.

Choline oxidase is a flavin-dependent enzyme with a dimeric structure and a mass of 120 kDa (*4*). The enzyme has gained attention from research field because of its almost unique catalytic strategy, which allowed the oxidation of an alcohol and an aldehyde to occur in the same active site. The detail catalytic reaction of choline oxidase is illustrated in Scheme 5.1, which involves the oxidation of choline to glycine betaine through two oxidation steps with betaine aldehyde as the intermediate and molecular oxygen as the final electron acceptor (Scheme 5.1).



Scheme 5.1. Reaction catalyzed by choline oxidase.

Other than the interesting catalytic mechanism, choline oxidase was considered of importance to both medical and biotechnological applications because the product of its catalytic reaction,

glycine betaine. Glycine betaine is a compatible solute that can be found to accumulated by (*5, 6*) human pathogens (*7-16*) and crop plants (*17-22*) as a response to dehydration and plasmolysis in the environments. For these reasons, the study of choline oxidase can give insights into glycine betaine biosynthesis found in bacterial and plants, enabling the development of drug that can target the resistance of human pathogens to the environments on host tissue and for the engineering of transgenic plants that carried drought and salt stress resistance.

Over the years, our group have reported a number studies on choline oxidase. Firs, the cloning and expression of the gene coding for choline oxidase from *A. globiformis* strain ATCC 8010 in *E. coli* was carried out successfully (*4*). Kinetic isotope effect was used to elucidate the chemical mechanism for the oxidation of choline catalyzed by choline oxidase (*23, 24*). The results suggested that the hydride transfer between the α-carbon of the activated alkoxide form of choline and the enzyme-bound flavin cofactor occurred quantum mechanically. The presence of an active site base with pK_a of ~7.5 was also established based on the data from pH and kinetic isotope effects of the kinetic parameters of choline oxidase with choline as substrate (*25-27*). The roles of His466 was characterized when the histidine was replaced with and alanine or an asparatate. Spectroscopic and kinetic properties of H466A and H466D mutants suggested that His466 stabilized in the negative charges in the enzyme active site by its imidazolium side chain and contributed into the polarity of the microenvironment of the flavin (*28, 29*).

Despite the in depth understanding on the catalytic mechanism of choline oxidase based kinetic and mechanistic studies, our investigation on this enzyme is still limited without 3-dimensional structure. Consequently, chemical modification of choline oxidase using tetranitromethane was used in this study to identify the tyrosine residue that may be essential for

catalysis. Kinetic and structural properties of the inactivation of choline oxidase by tetranitromethane were characterized using spectrophotometric and chromatographic techniques.

Experimental Procedures

Materials and enzyme preparations. Choline chloride was from ICN Pharmaceutical (Aurora, OH). Glycine betaine and tetranitromethane were from Sigma-Aldrich (St. Louis, MO). TPCK-treated trypsin was purchased from Worthington (Lakewood, NJ). Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described previously (*4*). Fully oxidized choline oxidase was prepared as described in ref. (*27*), and was stored at -20 ^{o}C in either 20 mM Tris-Cl, pH 8, or 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6. The enzyme was rapidly thawed on ice prior of each experiment. The concentration of choline oxidase was determined spectrophotometrically at 454 nm using the previously reported ε_{454} value of 11.4 $mM^{-1}cm^{-1}$ (*26*). All kinetic parameters determined were expressed per active site oxidized flavin content (*26*). All other reagents were of the highest purity commercially available.

Instruments. Inactivation of enzyme was carried out by incubating the enzyme with the inhibitor in Eppendorf Mastercycler model 5333 thermostated at 20 ^{o}C. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode (HansaTech Oxy-32) thermostated at 25 ^{o}C. UV-visible absorbance spectra were recorded on a Hewlett-Packard 8453A diode array spectrophotometer. Purification of peptides was carried out by HPLC using a Shimadzu instrument equipped with a model SPD-M10A photodiode array detector and a μBondapak C18 (15 × 4.6 mm) reverse-phase column.

Enzyme assays. Enzyme activity was measured with the method of the initial rates (*30*) in air-saturated 50 mM potassium phosphate at pH 7 by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 25 ^{o}C. The reactions were started by the addition of choline

oxidase to a 1 ml reaction mixture. Unless otherwise stated, the final concentration of enzyme in the reaction mixture was in 0.1 to 0.5 μM range, and the concentration of choline was 10 mM. The enzyme was inactivated by incubating in the presence of tetranitromethane in 20 mM Tris-Cl at pH 8 and 20 ^{o}C. At different times, aliquots were withdrawn and assayed polarographically for enzymatic activity. Stock solution of tetranitromethane was prepared in ethanol prior use.

Tryptic Digestion. The enzyme was first incubated with tetranitromethane in the presence and absence of 140 mM glycine betaine until there was ~10% of residual activity left in the unprotected sample. The enzyme was isolated from excess TNM by gel filtration. The inactive enzyme was denatured with 10% (w/v) trichloroacetic acid and incubated on ice for 30 min before centrifugation at 12000*g* for 20 min. The pellets were washed with 10% trichloroacetic acid followed by an ice-cold acetone/HCl (39:1) mixture, resuspended in 0.1 mL of 8 M urea, 4 mM $CaCl_2$, and 0.4 M ammonium bicarbonate, pH 8, and allowed to stand for 1 h at 37 °C. The solutions were then diluted with 0.3 mL of water before the addition of trypsin to a final concentration of 3% (w/w, trypsin/choline oxidase). After 4 h incubation at 37 °C, a second aliquot of trypsin (1% w/w final concentration) was added, and the mixture was allowed to react for a further 15 h at 37 °C. The digestion was stopped with trifluoroacetic acid (1% final concentration). Purification of peptides was carried out by HPLC using a Shimadzu instrument equipped with a model SPD-M10A photodiode array detector and a μBondapak C18 (15 × 4.6 mm) reverse-phase column at a flow rate of 1 mL min^{-1}. Eluent A was 0.05% aqueous trifluoroacetic acid, and eluent B was 0.04% trifluoroacetic acid in acetonitrile. The chromatography was carried out with a linear gradient from 5% to 50% eluent B over 90 min. Peptide fragments were collected manually. MALDI-TOF mass spectrometry in both the

positive and negative ion mode with 50:50 methanol/acetonitrile matrix was used to identify the modified peptide.

Data Analysis. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA). The steady state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation for one substrate. Time course of inactivation of choline oxidase was fit with eq (1), where A represents the residual activity at a given time (t), A_0 is the initial activity, and k_{obs} is the observed rate of the inactivation. The rates of inactivation of the enzyme were fit to eq (2), where k_{inact} is the first-order rate constant for the conversion of the reversibly formed enzyme-inhibitor complex to irreversibly inactivated enzyme, [I] is the concentration of the mechanism-based inhibitor, and K_I is the dissociation constant of the mechanism-based inhibitor from the enzyme.

$$A = A_0 e^{-k_{obs} t} \qquad (1)$$

$$k_{obs} = \frac{k_{inact}[\mathrm{I}]}{[\mathrm{I}] + K_I} \qquad (2)$$

Results

Inactivation of Choline Oxidase with Tetranitromethane. When choline oxidase was incubated with tetranitromethane in 20 mM Tris-Cl at pH 8 and 20 ^{o}C, a time-dependent loss of enzymatic activity was observed (Figure 5.1 panel A). The inactivation was dependent on the concentration of tetranitromethane. When the observed rates of inactivation were plotted as a function of the concentration of tetranitromethane (Figure 5.1 panel B), a saturation curve was observed, suggesting the inactivation followed first-order kinetics that involved reversible binding of tetranitromethane to the enzyme followed by irreversible inactivation of the enzyme. The curve was fit with equation 2 to yield a k_{inact} of 0.32 ± 0.01 h^{-1} and a K_I of 0.66 ± 0.04 mM. In the presence of glycine betaine, the enzyme was fully protected from inactivation at ≤2.5 mM tetranitromethane, but it was only partially protected at >2.5 mM tetranitromethane, suggesting that the inactivation might become nonselective at high concentration of tetranitromethane. No activity recovery was detected when the inactivated enzyme was isolated by gel filtration then incubated in 20 mM Tris-Cl at pH 8 and 20 ^{o}C for 2h. This is consistent with the inactivation choline oxidase by tetranitromethane being irreversible. To verify that the presence of tetranitromethane did not affect the biological function of active choline oxidase, the enzyme was inactivated to 30% of residual activity then isolated by gel filtration. Activity assay of this sample yielded a turn over number (k_{cat}) of 7.6 ± 0.1 s^{-1}, which was equivalent to ~30% of the k_{cat} value of the fully active enzyme, i. e., 18.6 ± 0.6 s^{-1} (Figure 5.2). The Michaelis-Menten constant (K_m) in partially modified and native enzyme samples were 0.61 ± 0.03 mM and 0.63 ± 0.07 mM, respectively, suggesting that the activity detected was only due to the active form of the enzyme in the partially modified sample. In this context, the data indicated that the partially

modified enzyme sample contains a mixture of inactive and active enzymes and that the active enzyme in the sample was still functioned normally and was capable of catalysis.

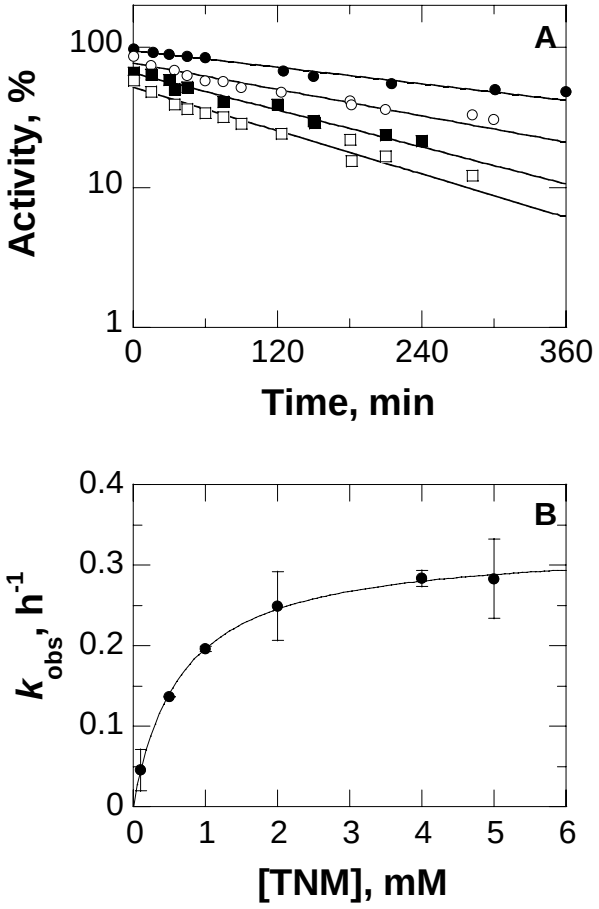


Figure 5.1. Time-dependent inactivation of choline oxidase with tetranitromethane. Choline oxidase at 0.54 μM was incubated with different concentrations of tetranitromethane in 20 mM Tris-Cl, pH 8, at 20 ^{o}C. Enzyme activity was monitored over time in 50 mM potassium phosphate, pH 7, at 25 ^{o}C. (A) Time course of inactivation at different concentrations of tetranitromethane. For clarity, only data with (●) 0.5 mM, (○) 1.0 mM, (■) 4 mM, and (□) 5 mM tetranitromethane are shown. (B) Secondary plot of the observed rate of inactivation as a function of the concentration of tetranitromethane. The data were fit to equation (2).

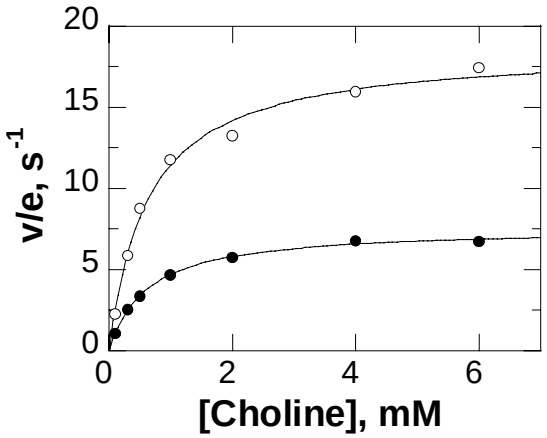


Figure 5.2. Activity assay of native (○) and TNM-modified (●) choline oxidase in 50 mM potassium phosphate, pH 7, at 25 °C. The TNM-modified enzyme sample was prepared by incubating the enzyme with 5 mM TNM in 20 mM Tris-Cl, pH 8, at 20 °C. Inactivation of enzyme was allowed to occur until 35% of residual activity remained, then excess TNM was removed using gel filtration. The data were fit to Michaelis-Menten equation for one substrate.

Properties of Choline Oxidase upon Inactivation with Tetranitromethane. The TNM-modified form of choline oxidase in the presence and absence of glycine betaine was isolated with gel filtration and their UV-visible absorbance spectra were compared (Figure 5.3). Both spectra showed a significant increase in absorbance at 360 nm as compared to the spectrum of native enzyme, indicating the presence of nitrotyrosines. However, the TNM-modified sample in the absence of glycine betaine has spectrum with larger intensity as compared to that of the enzyme sample that was incubated in the presence of glycine betaine, suggesting a different amount of modified tyrosine in the enzyme when it was partially protected from inactivation. The amount of tyrosine residues being modified was quantitated using the absorbance difference between the TNM-treated enzyme and the native enzyme and ε_{360} = 14 M^{-1} cm^{-1} for the nitrated tyrosine at 360 nm (*2, 31*). The result showed that there was a difference of 5 modified tyrosyl residues in TNM-modified enzyme sample in the absence of glycine betaine as compare to the TNM-modified sample in the presence of glycine betaine.

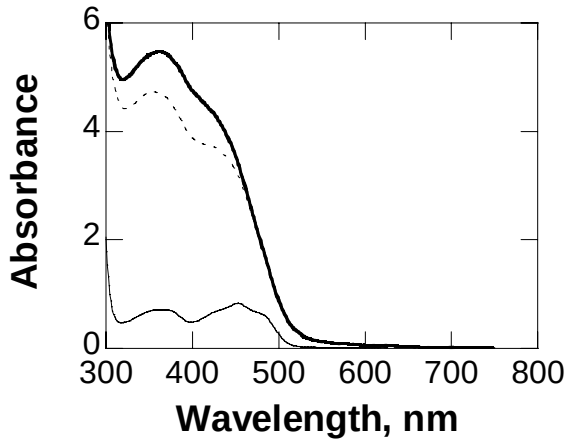


Figure 5.3. UV-visible absorbance spectra of the native form (thin line) and the TNM-modified forms of choline oxidase in the presence (dotted line) and in the absence (thick line) of glycine betaine. Both protected and unprotected enzyme samples were incubated with 8 mM tetranitromethane in 20 mM Tris-Cl at pH 8 and 20 ^{o}C.

Identification of the Site of Modification of Choline Oxidase by Tetranitromethane. To identify the site of modification by tetranitromethane that might be responsible for the inactivation of choline oxidase, the enzyme was inactivated with tetranitromethane to 24% residual activity. Excess tetranitromethane was removed by gel filtration. The sample was digested with trypsin into peptide fragments. The peptides were separated by reverse-phase HPLC and compared to the peptides of another enzyme sample that was treated with the same concentration of tetranitromethane but was partially protected by glycine betaine (Figure 5.4). The two chromatographic maps at 360 nm contained various differences both in peak intensity and position. The peptides eluting at 31.1 min were collected because the peak at this retention time showed the most significant difference. Mass spectrometric analysis showed that the same peptide fragments were found in both protected and unprotected sample (data not shown), suggesting nitration at these peptides might not be responsible for the inactivation of choline oxidase by tetranitromethane.

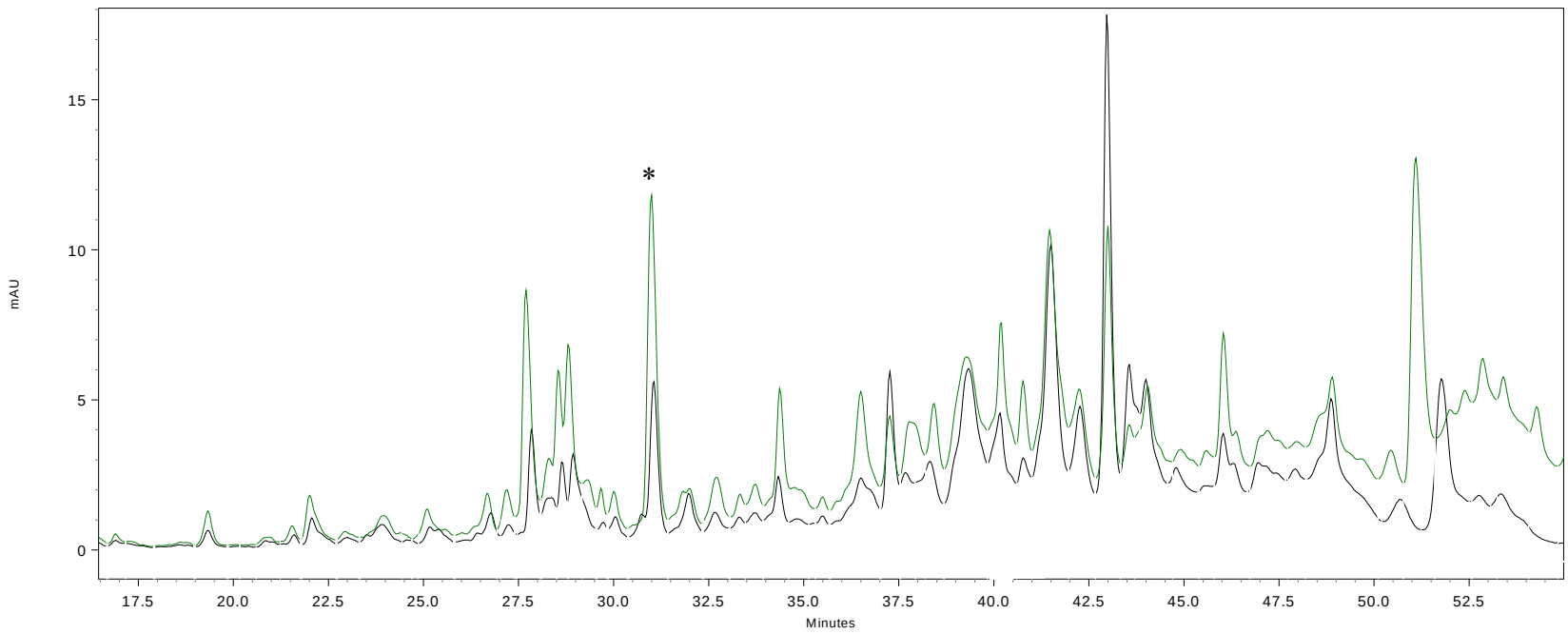


Figure 5.4. Overlaid HPLC chromatograms of tryptic digests of choline oxidase treated with tetranitromethane in the presence (black line) and absence (green line) of glycine betaine. Peptide elution was monitored at 360 nm with a photodiode array spectrophotometer detector. The peak that eluted at 31.1 min (*) was collected for mass spectrometry analysis.

Discussion

The biosynthesis of glycine betaine by choline oxidase is of importance to both medical and biotechnological applications because glycine betaine was found to accumulate in beneficial plants and bacterial as stress resistance to adverse hyperosmotic environments (*7-22*). Over five years, detailed studies on kinetics and mechanistic properties of choline oxidase were carried out, providing a good understanding on the catalytic mechanism of the enzyme. However, in the absence of crystallographic data, the study on catalytic strategy of choline oxidase at structural level was limited. An alternate approach using chemical modification was employed in order to identify essential catalytic residue in the active site of choline oxidase. In this study, tetranitromethane was chosen for the modification study of choline oxidase because it can inactivate the enzyme with significant rate.

The results presented herein show that the inactivation of choline oxidase with tetranitromethane is both time and concentration dependent. The evidence for this conclusion comes from the single exponential decay of enzymatic activity in the presence of tetranitromethane. The observed rate of inactivation increased with increasing concentration of tetranitromethane to a limiting value. This is consistent with the reversible binding of enzyme and tetranitromethane occurred before the inactivation of the enzyme. Evidence for the inactivation of the enzyme being irreversible was found when the isolated inactive enzyme was incubated at 20 ^{o}C and no activity recovery was observed for 3 h.

The inactivation of choline oxidase is active-site directed with ≤2.5 mM tetranitromethane but is nonselective with >2.5 mM tetranitromethane, as suggested by the protective effect of glycine betaine on the enzyme. The increase in intensity of UV-visible absorbance at 360 nm of the TNM-modified enzyme is the evidence for formation of nitrated

tyrosine upon inactivation with tetranitromethane. The fact that the nitration of tyrosine was observed in both protected and unprotected enzyme sample can be explained by the fact that choline oxidase has 11 tyrosyl residues in its amino acid sequence, so it is likely that tyrosyl residues outside the active site have been modified. Such modification might be responsible for the partial inactivation of the enzyme in the presence of a ligand in the active site. This is consistent with the data from UV-visible absorbance spectra, which showed that the unprotected enzyme sample has 5 nitrotyrosines more than that found in the protected sample when the inactivation was carried out with 8 mM tetranitromethane.

Comparison of the tryptic maps of protected and unprotected enzyme sample revealed the nonspecific modification of tetranitromethane, as suggested by various differences found on the chromatograph at 360 nm. Since the enzyme was only partially protected at tetranitromethane >2.5 mM, the results cannot be improved by using high concentration of tetranitromethane.

In conclusion, the chemical modification of choline oxidase using tetranitromethane resulted in nonselective modification; therefore, no essential peptide can be identified in this study. Future study will be necessary to obtain a more selective modification of choline oxidase using different mechanism-based inhibitor. The identification of a residue that is of importance to the catalytic mechanism of choline oxidase can be used in future mutagenesis to better understand the catalytic strategy of the enzyme.

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Chapter VI

TRAPPING OF CHOLINE OXIDASE IN A NON-FUNCTIONAL CONFORMATION BY FREEZING AT LOW PH

(A revised version of this chapter has been submitted for publication in *Proteins* by Hoang, J. V. and Gadda, G. on July 2006)

Abstract

Choline oxidase is a flavin-dependent enzyme that catalyzes the oxidation of choline to glycine-betaine, with oxygen as electron acceptor. Storage at pH 6 and -20 ^{o}C resulted in a change in the conformation of choline oxidase, as suggested by spectroscopic data, which was associated with compete loss of catalytic activity when the enzyme was assayed at pH 6. Treatment of the inactive enzyme at pH values ≥6.5 and 25 ^{o}C resulted in the fast and partial reactivation of the enzyme, which occurred within 6 min, resulting in the slow onset of steady state during enzymatic turnover. The rate of approaching steady state was independent of the concentrations of choline and enzyme, but increased to a limiting value with increasing pH, defining a macroscopic pK_a value of ~7.3 for an unprotonated group required for enzyme activation. Prolonged incubation of the inactive enzyme at pH 6 and temperatures ≥20 ^{o}C resulted in the slow and full recovery of activity over 3 hours, associated with a conformational change that reverted the enzyme to the native form, as suggested by spectrophotometric data. The temperature-dependence of the slow enzyme activation at pH 6 yielded $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values of ~112 kJ mol^{-1} and ~64 J K^{-1} mol^{-1}, respectively, resulting in an entropy of activation (T$\Delta S^{\ddagger}$) at 25 ^{o}C of ~20 kJ mol^{-1}. These data suggest that freezing the enzyme at low pH induces a localized and reversible conformational change that is associated with the complete and reversible loss of catalytic activity.

Introduction

For years, freezing has been used extensively for the storage of purified enzymes. In most cases, isolated soluble enzymes stored at sub-freezing temperatures and neutral or slightly alkaline pH values are found to be stable and maintain catalytic activity, although in some cases partial loss of catalytic activity due to the unfolding of the protein may arise during the freezing and thawing processes. In a limited number of cases, however, subjecting purified enzymes to storage at sub-freezing temperatures results in the reversible loss of catalytic activity not being associated with unfolding of the protein, as exemplified by L-amino acid oxidase, phosvitin, and glutamate mutase (*1-8*). In this context, significant deviations of the pH from neutrality in the storage buffer have been shown to contribute to inactivation, due to changes either in the quaternary structure of the protein or in the ionization of groups that participate in catalysis (*9*). For the documented cases of ovoperoxidase, phosphofructokinase, leucocyte proteinase 3, and formyltetrahydofolate synthetase, such pH effects on the catalytic activity of the enzyme have been shown to be reversible (*9-12*). A common manifestation of the reversibility of the inactivation process is a slow onset of steady state during enzymatic turnover (*10, 12*). While such a hysteretic behavior is commonly found in allosteric enzymes as a strategy for metabolic regulation (*13-16*), it is not common for other classes of enzymes. During the procedure employed to purify choline oxidase, the enzyme showed a significant hysteretic behavior after storing at pH 6 and -20 ^{o}C (Fan Fan, Prashanthi Menon, Jane V. Hoang, and Giovanni Gadda; unpublished observations), a step used to fully oxidize the enzyme-bound flavin[4] (*18*), suggesting that the enzyme might be affected by freezing at low pH.

[4] The enzyme-bound flavin in choline oxidase as purified is present as a mixture of oxidized and anionic flavosemiquinone *(17)*. The fully oxidized enzyme is commonly prepared by extensive dialysis at pH 6 and 4 ^{o}C over 24 hours, as reported in ref. *(18)*, or by short dialysis at

Choline oxidase (E.C. 1.1.3.17) catalyzes the flavin-mediated two-step oxidation of choline to glycine betaine, with betaine aldehyde as intermediate and molecular oxygen as electron acceptor (Fig. 1) (*19-21*). The enzyme is of both biotechnological and medical interest since glycine betaine can be accumulated in the cytoplasm of cells to prevent dehydration and plasmolysis in adverse hyperosmotic environments (*22, 23*) in pathogenic bacteria (*24-33*) and transgenic plants (*34-39*). Consequently, the study of choline oxidase has potential for the development of therapeutic agents targeted at the inhibition of glycine betaine biosynthesis and for the engineering of drought resistance in economically relevant crops. Furthermore, choline oxidase has been extensively used for the development of biosensors to detect choline and choline ester derivatives, such as acetylcholine, in serological fluids and foodstuff (*40-44*). The enzyme is a homodimer of 120 kDa, with each monomer containing covalently bound FAD (*17*). The chemical mechanism for the oxidation of choline catalyzed by choline oxidase has been recently elucidated using kinetic isotope effects, and is consistent with the quantum mechanical transfer of a hydride from the substrate α-carbon to the enzyme-bound flavin cofactor occurring from an activated alkoxide form of choline (*45, 46*). Such an alkoxide species has been proposed to form by the action of an active site base with pK_a of ~7.5 (*18, 47, 48*), and to be electrostatically stabilized in the enzyme active site by the imidazolium side chain of His_{466} (*49, 50*). In the present study, we have investigated the effect of storage temperature and pH on the biophysical and catalytic properties of choline oxidase. The results of these studies suggest that freezing the enzyme at low pH induces a localized and reversible conformational change that is associated with the complete and reversible loss of enzymatic activity.

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pH 6 and 4 ^{o}C over 3 to 5 hours, followed by storage at pH 6 and -20 ^{o}C (Fan Fan, Prashanthi Menon, Jane V. Hoang, and Giovanni Gadda; unpublished observations).

Experimental Procedures

Materials and enzyme preparations. Choline chloride was from ICN Pharmaceutical (Aurora, OH). Glycine betaine was from Sigma-Aldrich (St. Louis, MO). Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described previously (*17*). Fully oxidized choline oxidase was prepared as described in ref. (*18*), and was stored at -20 ^{o}C in either 20 mM Tris-Cl, pH 8, or 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6. The enzyme was rapidly thawed on ice prior of each experiment. When needed, buffers were changed either by gel filtration through disposable PD-10 desalting columns (Amersham Pharmacia Biotech, Piscataway, NJ) or dialysis for 19 h at 4 ^{o}C. The concentration of choline oxidase was determined spectrophotometrically at 454 nm using the previously reported ε_{454} value of 11.4 $mM^{-1}cm^{-1}$ (*48*). All kinetic parameters determined were expressed per active site oxidized flavin content (*48*). All other reagents were of the highest purity commercially available.

Instruments. UV-visible absorbance spectra were recorded using an Agilent Technologies diode-array spectrophotometer Model HP 8453; fluorescence spectra were acquired on a Shimadzu spectrofluorometer model RF-5301; circular dichroic spectra were determined using a Jasco J-180 spectropolarimeter. Time-courses of oxygen consumption were determined polarographically with an oxygen electrode from Hansatech Instruments Ltd. (Norfolk, UK).

Spectral studies. Absorbance, fluorescence, and circular dichroic spectra were determined in 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6, at 15 ^{o}C. The concentration of enzyme in flavin content was: 11 μM for the UV-visible absorbance spectra, 3 μM for the protein fluorescence emission spectra (with λ_{ex} at 285 nm), 11 μM for the flavin

fluorescence emission (with λ_{ex} at ~455 nm), and 2.5 μM, 50 μM, and 168 μM for the CD spectra of the protein in the far-UV, near-UV, and visible regions of the spectra, respectively.

Enzyme assays. Unless otherwise stated, time-courses of oxygen consumption and enzymatic activity were determined polarographically by measuring the rate of oxygen consumption using a computer-interfaced oxygen electrode system in air-saturated 50 mM potassium phosphate, at pH 7 and 25 ^{o}C. The determination of the kinetic parameters of choline oxidase was carried out using the method of the initial rates (*51*), with concentrations of choline in the range from 0.1 to 10 mM. For the determinations at pH 8 and 9, 50 mM sodium pyrophosphate was used as buffer. For the enzyme species showing slow onsets of steady state during turnover, rates of oxygen consumptions were determined from the linear portions of the traces, i.e., after steady state was achieved.

Effect of pH on hysteresis. For the determination of the pH effects on the fast rate of reactivation of choline oxidase (k_c), the activity of the enzyme species with hysteretic behavior was measured in 50 mM Tris (hydroxymethyl) aminomethane with the pH in a range from 6.5 to 9.6 at 25 ^{o}C.

Effect of temperature on reactivation of choline oxidase at pH 6. The temperature effects on the slow rate of activation of choline oxidase (k_{act}) were determined by incubating the inactive enzyme in the range from 20 to 39 ^{o}C at pH 6, and the activity was measured in 50 mM morpholinoethansulfonic acid, at pH 6 and 25 ^{o}C.

Data analysis. Data were fit with KaleidaGraph software (Biosoft, Cambridge, UK). The steady state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation for one substrate. The first-order rate constants for the fast and pH-dependent activation of the inactive form of choline oxidase (k_c) were determined by fitting the time-courses of

oxygen consumption to eq 1, which was modified from Frieden (*16*), where v_f represents the observed rate of reaction after reaching steady state, and $[O_2]_o$ is the initial concentration of oxygen. The pH dependence of k_c was determined by using eq 2, which describes a curve with a slope of +1 and a plateau region at high pH where C represents the pH-independent value. The apparent rates of activation of the enzyme at any given temperature were determined by fitting the observed rates of oxygen consumption at any given incubation time with eq 3 that describes a single exponential process. Here, ${}^{app}k_{act}$ is the apparent first-order rate constant for the temperature-dependent slow activation of the inactive form of choline oxidase, t is time, A_t is the value of enzymatic activity, A_o is the amplitude of the total change, and A_∞ is the enzymatic activity at infinite time. The temperature dependence of k_{act} was determined by using the Eyring's equation (eq 4), where k_{act} is the first-order rate constant for enzyme activation, k_B and h are the Boltzmann and Planck constants, T is the absolute temperature in Kelvin, R is the gas constant with a value of 8.315 J mol^{-1} K^{-1}, and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively.

$$\Delta[O_2]_t = v_f t - v_f \frac{1-e^{-k_c t}}{k_c} + [O_2]_o \qquad (1)$$

$$\log k_c = \log\left(\frac{C}{1+\frac{10^{-pH}}{10^{-pKa}}}\right) \qquad (2)$$

$$A_t = A_\infty - A_o e^{-t\,{}^{app}k_{act}} \qquad (3)$$

$$\ln\left(\frac{k_{act}}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^{\ddagger}}{R} - \frac{\Delta H^{\ddagger}}{RT} \qquad (4)$$

Results

Effect of storage pH and temperature on the enzymatic activity of choline oxidase. Storage of the enzyme at pH 6 and -20 ^{o}C resulted in a choline oxidase form that was devoid of enzymatic activity at pH 6 and 25 ^{o}C, as suggested by the lack of oxygen consumption over extended incubation of the enzyme with 1 mM choline in 50 mM potassium phosphate, pH 6. In contrast, this enzyme form slowly regained enzymatic activity with 1 mM choline at pH 7 and 25 ^{o}C, with a significant hysteretic behavior that was apparent as a slow onset of steady state during enzymatic turnover (Figure 6.1).

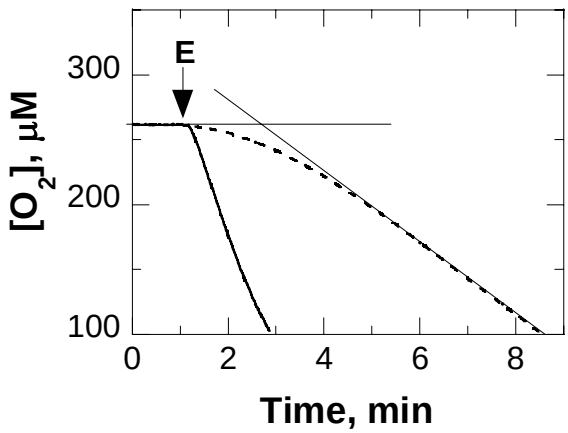

Figure 6.1. Time-courses of oxygen consumption during turnover of choline oxidase at a final concentration of 0.54 µM with 1 mM choline as substrate in air-saturated 50 mM potassium phosphate, pH 7, at 25 ^{o}C. Solid curve represents the trace using choline oxidase previously stored at pH 8 and -20 ^{o}C; dashed curve is the trace using choline oxidase previously stored at pH 6 and -20 ^{o}C. **E** indicates the time at which the enzyme was added to the assay reaction mixture. Thin lines represent the tangents to the linear portions of the trace obtained with choline oxidase previously stored at pH 6 and -20 ^{o}C.

Significant lags in catalytic turnover were also observed when the enzyme was flash-frozen in a mixture of dry ice and acetone at pH 6. In contrast, no lag in the time-courses of oxygen consumption was observed when the enzyme was stored at pH 6 and 0 ^{o}C or at pH 8, irrespective of whether the storage temperature was -20 ^{o}C or 0 ^{o}C. Similarly, no hysteretic behavior was

seen when the enzyme was stored at pH 6 and -20 ^{o}C or -80 ^{o}C in the presence of 30 or 50% glycerol as anti-freezing agent, or when choline oxidase was kept on ice for as long as 26 h. These data suggest that freezing of choline oxidase at low pH results in a form of enzyme with altered catalytic properties (*E′*).

To evaluate whether the presence of a ligand in the active site of the enzyme affected the formation of *E*', choline oxidase was frozen at pH 6 in the presence of 0.14 M glycine betaine, which resulted in ~90% of the enzyme being in a glycine betaine-complex at this pH (*48, 50*). The time-courses of oxygen consumption of the enzyme stored in the presence of glycine betaine showed hysteretic behavior (data not shown), suggesting that the altered *E*' form of choline oxidase could be formed irrespective of whether the enzyme was in the free or liganded form.

Kinetic parameters of choline oxidase stored at pH 6 and -20 ^{o}C. The turnover number (k_{cat}) and the Michaelis-Menten constant (K_m) for choline as substrate for the enzyme previously stored at pH 6 and -20 ^{o}C were determined in 50 mM potassium phosphate, at pH 7 and 25 ^{o}C, and were compared to those determined for the enzyme that did not show hysteretic behavior (*E*), which was prepared in three different ways by storing choline oxidase at pH 6 or 8 and 0 ^{o}C, or at pH 8 and -20 ^{o}C. As shown in Table 6.1, *E*' showed a k_{cat} value that was 3-times lower than the value determined for *E*, and a K_m value that was similar to that determined for *E*. Kinetically, these data suggest that the altered form of choline oxidase prepared by freezing at pH 6 was devoid of enzymatic activity, and that upon reaching steady state at pH 7 and 25 ^{o}C the enzyme existed as a mixture of the fully active form *E* that is responsible for the consumption of oxygen (~30%) and the inactive form *E*'. Similar results with k_{cat} values that were ~30% of the value determined for the native enzyme and K_m values that were similar to those of the native enzyme were observed with the altered form of choline oxidase at pH 8 and 9 (data not shown),

suggesting that only 30% of the inactive enzyme could regain activity within the time required for the oxygen electrode recordings (3 to 6 min).

Table 6.1. Kinetic parameters of choline oxidase stored at pH 6 and -20 °C with choline as substrate at pH 7 and 25 °C. [a]

storage pH	storage temperature, °C	k_{cat}, s^{-1}	K_m, mM	L [b], s
6	-20	4.4 ± 0.1	0.7 ± 0.1	30 – 70
6	0	13.3 ± 0.2	0.5 ± 0.1	nd [c]
8	0	14.9 ± 0.2	0.6 ± 0.1	nd
8	-20	15.5 ± 0.1	0.6 ± 0.1	nd

[a]The enzymatic activity of choline oxidase that was previously stored at the pH and temperature indicated was measured at varying concentrations of choline in air-saturated 50 mM potassium phosphate, pH 7, at 25 °C.
[b]*L* represents the length of the lag phase observed before the rate of oxygen consumption reached steady state.
[c] nd, not detectable.

Effect of choline and enzyme concentrations on hysteresis. The effect of choline concentration on the hysteretic behavior of choline oxidase under turnover was determined with the *E′* form of enzyme at a final concentration of 0.54 μM, in air-saturated 50 mM potassium phosphate, pH 7 and 25 °C. Fitting of the time-courses of oxygen consumption obtained with choline concentrations in the 0.5 to 8 mM range by using eq 1 yielded first-order rate constants for the attainment of steady state (k_c) that were independent of choline concentration (Table 6.2), with an average value of 0.017 ± 0.001 s^{-1}. In contrast, the time required to reach steady state, i.e., lag time, decreased with increasing concentrations of choline and reached a limiting value of ~30 s, consistent with the apparent length of the lag phase in catalysis being inversely dependent on substrate concentration (Table 6.2).

The effect of enzyme concentrations in the range from 1.1 to 2.8 μM on the hysteretic behavior of choline oxidase was also determined with 1 mM choline as substrate at pH 7 and 25 ^{o}C. As summarized in Table 6.2, the k_c values were similar to each other and, most importantly, were not significantly different from the average k_c value determined at varying concentrations of choline. As for the case of choline, the apparent length of the lag phase in catalysis was inversely dependent on the enzyme concentration, with shorter lags seen at increasing concentrations of choline oxidase. Finally, no differences in the length of the lag phase preceding steady state were observed in the time-courses of oxygen consumption in assay reaction mixtures at pH 7 and 25 ^{o}C containing KCl at concentrations as high as 180 mM (data not shown), suggesting that the activation of the E' form of choline oxidase did not depend on the ionic strength of the solvent.

Table 6.2. Effect of enzyme and substrate concentrations on hysteresis at pH 7 and 25 °C. [a]

E', μM	[choline], mM	L[b], s	v_f[c], μM s^{-1}	k_c[c], s^{-1}	R^2
0.54	0.5	54	0.83 ± 0.01	0.015 ± 0.001	0.9997
0.54	2.0	42	1.30 ± 0.01	0.015 ± 0.001	0.9995
0.54	4.0	42	1.40 ± 0.01	0.018 ± 0.001	0.9999
0.54	6.0	36	1.65 ± 0.01	0.016 ± 0.001	0.9997
0.54	8.0	30	1.40 ± 0.01	0.016 ± 0.001	0.9998
1.10	1.0	48	1.28 ± 0.01	0.013 ± 0.001	0.9996
1.66	1.0	42	1.88 ± 0.01	0.012 ± 0.001	0.9994
2.20	1.0	36	1.93 ± 0.01	0.015 ± 0.001	0.9994
2.76	1.0	30	2.23 ± 0.01	0.016 ± 0.001	0.9993

[a] Time-courses of oxygen consumption during turnover of choline oxidase previously stored frozen at pH 6 at the final concentrations indicated (E') with choline in 20 mM Tris-Cl, pH 7 and 25 °C, were fit to eq 1.
[b] L is the lag in catalytic turnover, i.e., the time required to the enzyme to reach steady state conditions.
[c] v_f represents the observed rate of reaction after reaching steady state; k_c is the first-order rate constant for approaching steady state.

Effect of pH on hysteresis. The effect of pH on the hysteretic behavior of choline oxidase was determined in the range from 6.5 to 9.6 at fixed concentrations of choline as substrate for choline oxidase and 25 ^{o}C. The substrate concentration was set equal to the K_m value at each pH, in order to avoid artifactual contributions arising from the dependence of the length of the lag phase that precedes steady state on the concentration of choline[5]. The k_c values were determined at each pH value by fitting the time-courses of oxygen consumption to eq 1. The log k_c value increased to a limiting value with increasing pH, yielding a pK_a value of 7.3 ± 0.1 for a group that must be unprotonated for activation of the enzyme (Figure 6.2).

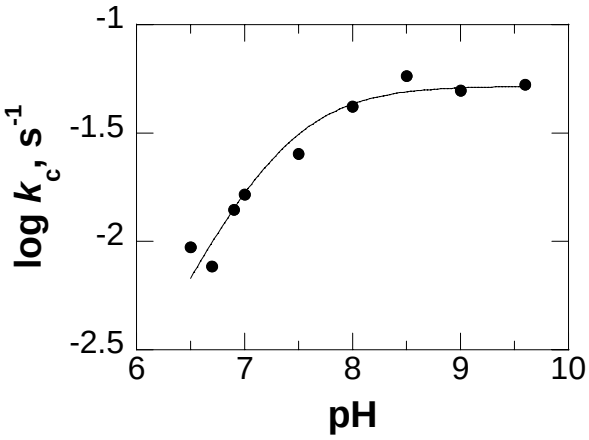

Figure 6.2. pH dependence of the first-order rate constant for approaching steady state (k_c) of choline oxidase previously stored at pH 6 and -20 ^{o}C. Enzymatic activity was determined with choline at concentrations set equal to the K_m values with 0.54 μM enzyme in 50 mM potassium phosphate at 25 ^{o}C. k_c values were determined by fitting the time-courses of oxygen consumption to eq 1. The curve is a fit of the data to eq 2.

Spectral properties of choline oxidase stored at pH 6 and -20 ^{o}C. In order to evaluate whether protein structural changes were associated with the different catalytic behavior of choline oxidase upon freezing at pH 6, the CD, absorbance, and fluorescence properties of *E′*

[5] Choline concentrations that were equal to K_m were chosen, instead of saturating concentrations, because at high pH and saturating concentrations of choline as substrate for the enzyme the lengths of the lag phases were too short in order to obtain accurate data.

were determined at pH 6 and 15 ^{o}C and compared to those of *E*. As shown in Figure 6.3A, the far-UV CD spectra of *E′* and *E* were virtually indistinguishable, consistent with both forms of choline oxidase being folded with similar contents in secondary structure elements. However, the UV-visible absorbance spectrum of *E′* showed a 9 nm hypsochromic shift of the peak centered at 366 nm and a 4 nm bathochromic shift of the band at 457 nm, both associated with significant decreases in intensities, with respect to the absorbance spectrum of *E* (Figure 6.3B). Both the protein and flavin fluorescence emission bands at ~340 nm and ~525 nm were two- to three-times more intense in the spectra of *E′* compared to *E* (Figure 6.3C-D). Furthermore, a significant bathochromic shift in the near-UV CD spectrum of *E′* was observed in the 270 to 290 nm region of the spectrum with respect to that of *E* (Figure 6.3E). Finally, an inversion of the polarity in the visible dichroic band in the 450 nm region of the CD spectrum of the enzyme-bound flavin was observed upon comparing the visible CD spectra of *E′* and *E*, as shown in Figure 6.4F. These data suggest that upon freezing the enzyme at low pH, choline oxidase undergoes a change in its conformation that significantly affects the flavin microenvironment.

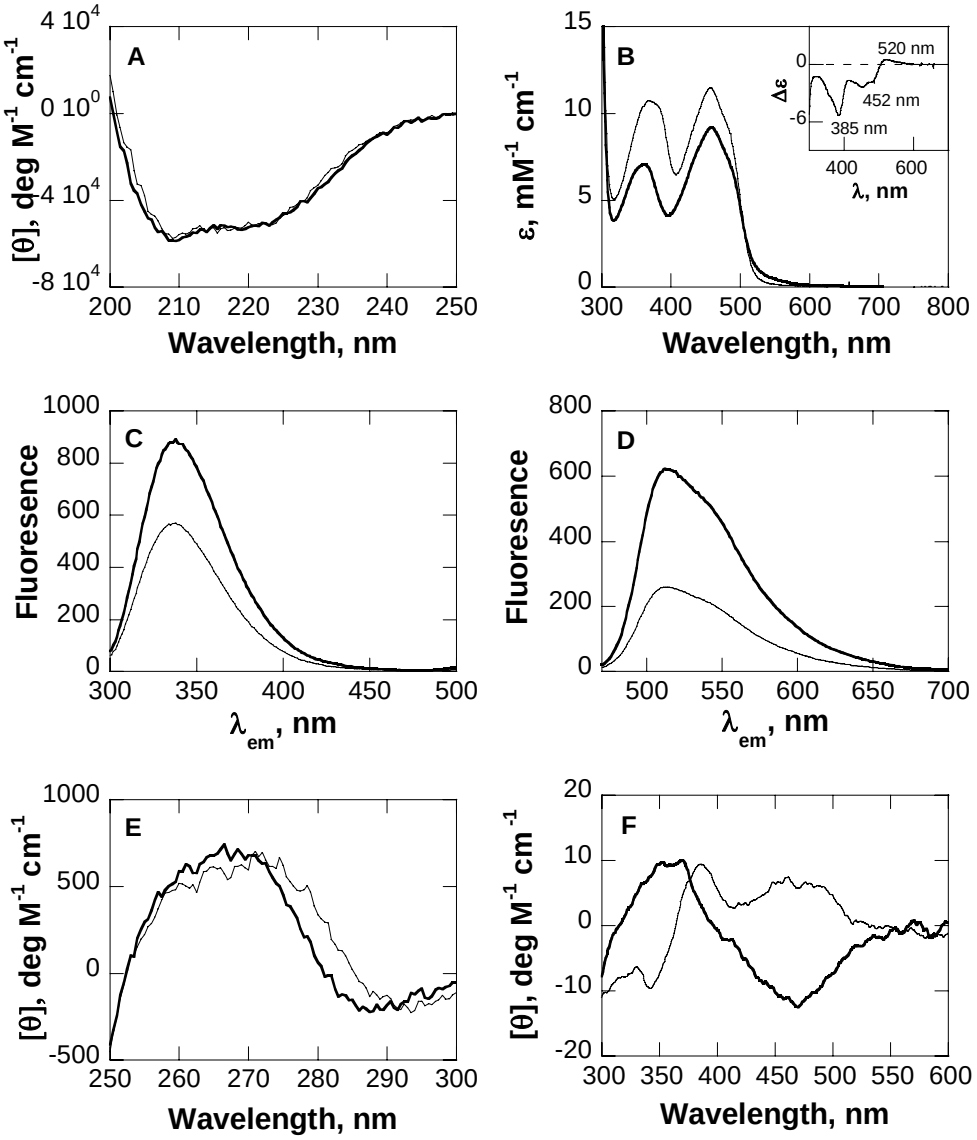

Figure 6.3. Determination of the apparent rate of activation of choline oxidase previously stored at pH 6 and -20 oC. The *E′* form of choline oxidase was incubated in 20 mM sodium phosphate, 20 mM sodium pyrophosphate, pH 6 and 25 ^{o}C, over 160 min. At different time intervals, aliquots of the enzyme solution were assayed for enzymatic activity with 1 mM choline as substrate in 50 mM 2-morpholinoethanesulfonic acid, pH 6 and 25 ^{o}C. Panel A, selected time-courses of oxygen consumption, where curves 1 and 6 represent the traces acquired after 2 and 72 min of incubation. Panel B, plot of the enzymatic activity as a function of incubation time for the determination of the first-order apparent rate constant for activation of choline oxidase by using eq 3.

Effect of temperature on activation of the E' form of choline oxidase at pH 6. The effect of temperature on the rate of activation of choline oxidase previously stored at pH 6 and -20 ^{o}C was determined at pH 6 with 1 mM choline as substrate. At this pH value, the first-order rate constant for the fast conversion of *E*' to *E* (k_c) was expected to be sufficiently slow (Figure 6.2) to affect minimally the time-course of oxygen consumption, thereby simplifying the analysis of the data. As expected, the time-courses of oxygen consumption did not show any lag before enzyme turnover reached steady state (Figure 6.4A). Moreover, as illustrated in the example of Figure 6.4 for the data at 25 ^{o}C, the rates of oxygen consumption increased to a limiting value that was similar to the value of ~5 s^{-1} obtained with 1 mM choline as substrate for the native enzyme with increasing time of incubation of the enzyme, yielding apparent rates for the slow temperature-dependent activation of the enzyme ($^{app}k_{act}$) at any given temperature (Table 6.3).

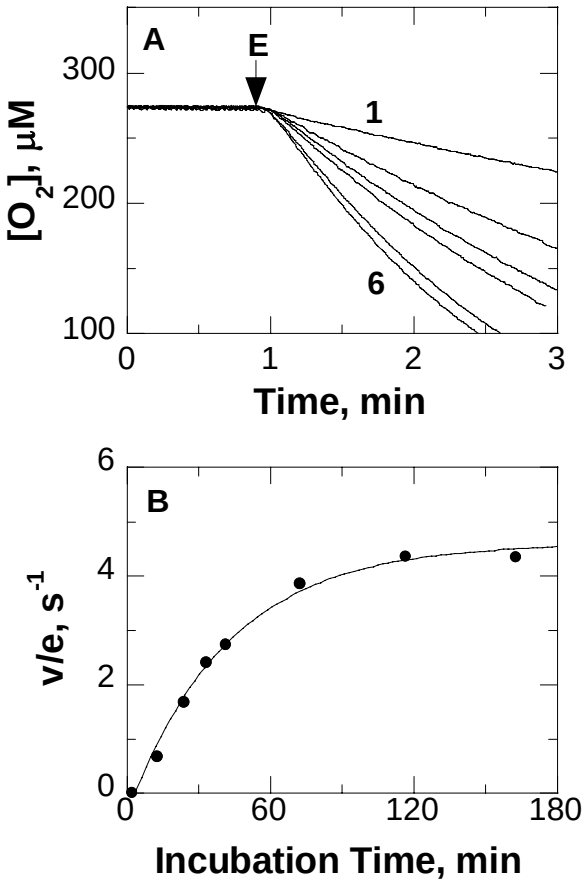

Figure 6.4. Temperature dependence of the first-order rate constant for activation of choline oxidase (k_{act}) in 20 mM sodium phosphate, 20 mM sodium pyrophosphate, at pH 6. The data were fit to eq 4.

Table 6.3. Effect of temperature on the rate of reactivation of choline oxidase at pH 6.[a]

temperature, ^{o}C	$^{app}k_{act}$ x 1,000 [b], s^{-1}	k_{den} x 1,000 [c], s^{-1}	k_{act} x 1,000 [d], s^{-1}
20	0.17 ± 0.01	0.04 ± 0.01	0.21 ± 0.01
25	0.47 ± 0.02	0.08 ± 0.01	0.55 ± 0.02
30	1.03 ± 0.11	0.04 ± 0.01	1.07 ± 0.11
33	1.87 ± 0.40	0.11 ± 0.01	1.98 ± 0.41
35	1.40 ± 0.22	0.28 ± 0.03	1.68 ± 0.22
37	2.50 ± 0.39	0.40 ± 0.06	2.90 ± 0.39
39	3.61 ± 1.17	0.53 ± 0.06	4.14 ± 1.17

[a] Enzymatic activity was determined with 1 mM of choline and 0.54 μM of enzyme in 50 mM air-saturated 2-morpholinoethanesulfonic acid, pH 6, at 25 ^{o}C.
[b] Measured rate of enzyme activation upon incubation of the *E′* form of enzyme at the indicated temperature.
[c] Observed rate of activity loss upon incubation of the fully active enzyme at the indicated temperature.
[d] True rate of enzyme activation, obtained by adding the rate of activity loss k_{den} to the measured rate of enzyme activation, $^{app}k_{act}$. The standard deviation for each of the k_{act} values was calculated from the square root of the sum of the squares of the standard deviations associated with k_{den} and $^{app}k_{act}$.

The contribution of enzyme denaturation ensuing from the prolonged incubations at high temperatures was taken into account by measuring the first-order rate constant for the loss of the enzymatic activity (k_{den}) of the fully active *E* form of choline oxidase (Table 6.3). These values were then added to the ${}^{app}k_{act}$ values in order to determine the true first-order rate constants for the activation of the *E′* form of choline oxidase in the temperature range from 20 to 39 °C (Table 6.3). An analysis of the k_{act} value according to the Eyring's formalism yielded a $\Delta H^{\ddagger}$ value of 112 ± 7 kJ mol^{-1} and a $\Delta S^{\ddagger}$ value of 64 ± 7 J K^{-1} mol^{-1} (Figure 6.5). These data are consistent with a $T\Delta S^{\ddagger}$ value of 19 ± 2 kJ mol^{-1} and a $\Delta G^{\ddagger}$ value of ~93 kJ mol^{-1} for the activation barrier for the conversion of *E′* to *E* at 25 °C. Such a small entropic contribution is consistent with the conformational change that is associated with the activation of the enzyme being localized rather than global.

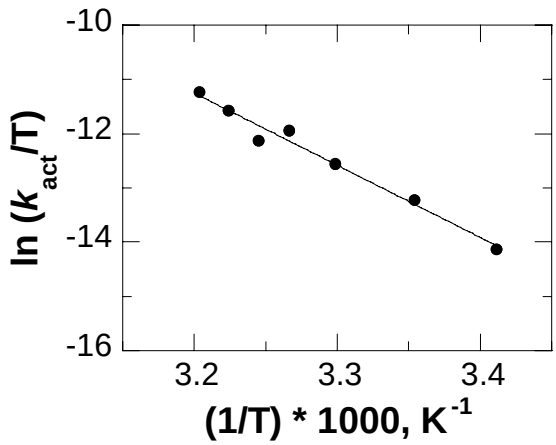

Figure 6.5. Temperature dependence of the first-order rate constant for activation of choline oxidase (k_{act}) in 20 mM sodium phosphate, 20 mM sodium pyrophosphate, at pH 6. The data were fit to eq 4.

As shown in Figure 6.6, incubation of the *E′* form of choline oxidase at pH 6 and 25 °C resulted in changes in the UV-visible absorbance spectrum of the enzyme, which slowly converted to that of the native form of choline oxidase. From the increase in absorbance at 385

nm associated with this process, an apparent first-order rate for the conversion of *E′* to *E* of (3.3 ± 0.3) x 10^{-4} s^{-1} was determined (Figure 6.6inset). The spectrophotometric changes observed suggest that the enzymatic activation of the altered form of choline oxidase is associated with a change in the conformation of the enzyme.

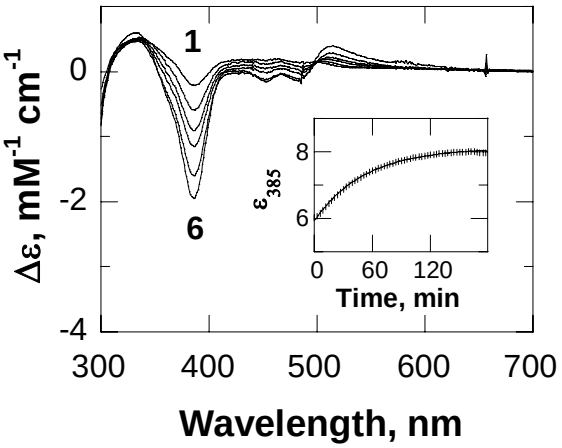

Figure 6.6. Conversion of the *E′* form to *E* form of choline oxidase. The *E′* form of choline oxidase was incubated in 20 mM sodium phosphate, 20 mM sodium pyrophosphate, at pH 6 and 25 ^{o}C, and UV-visible absorbance spectra were recorded over 180 min. Only selected spectra at different times of incubation minus the initial spectrum of the *E′ form* are shown; 1 and 6 refer to the difference spectra at 12 and 180 min of incubation. Inset, time-course of absorbance increase at 385 nm. Data were fit to eq 3.

Discussion

Choline oxidase is reversibly inactivated by freezing at pH 6. Evidence supporting this conclusion comes from the lack of oxygen consumption when the enzyme previously stored at pH 6 and -20 ^{o}C was assayed with choline as substrate at pH 6 and 25 ^{o}C. Upon raising the pH of the assay reaction mixture to values ≥6.5 the enzyme was slowly reactivated, as shown by the appearance of a detectable lag in the establishment of steady state during turnover with choline. Similar lags were also observed upon flash-freezing choline oxidase at pH 6 and -80 ^{o}C. In contrast, no lags in the time-course of oxygen consumption were observed upon storing the enzyme at pH 6 on ice or at sub-freezing temperatures in the presence of the anti-freezing agent glycerol. Furthermore, storage of the enzyme at pH 8 did not produce the altered catalytic behavior, irrespective of whether the temperature was 0 ^{o}C or -20 ^{o}C, indicating that inactivation of the enzyme results from the synergistic action of sub-freezing temperature and low pH. Similar effects of freezing on the catalytic activity of enzymes have been previously reported for a limited number of cases, such as L-amino acid oxidase (*1, 5-8*), glutamate mutase (*4*), and phosvitin (*2*).

From a biophysical standpoint, treatment of the enzyme at pH 6 and -20 ^{o}C induces a change in the conformation of choline oxidase, which is associated with the loss of catalytic activity. Evidence supporting a conformational change of the enzyme comes from the UV-visible absorbance, protein and flavin fluorescence emission, and near-UV and flavin circular dichroic spectra of choline oxidase after storage at -20 ^{o}C and pH 6, all showing significant differences with respect to the spectra of the enzyme stored at pH 8. In this respect, the altered conformation of the enzyme is not due to denaturation of the protein, since the far-UV circular dichroic spectroscopic analysis showed that the altered form of choline oxidase (*E*') maintains an overall

fold similar to that of the native enzyme. The circular dichroic spectrum of the flavin bound to the *E*' form of choline oxidase independently suggests that the enzyme is not denatured, since no circular dichroic features are expected for a flavin once it is extracted from the protein microenvironment (*52*). Mechanistically, the molecular motions that are required for the change in the conformation of choline oxidase might occur in the rigidity of the ice lattice, for which movements of photons, electrons, and protons for molecular reactions in frozen systems have been previously established (*53-56*). Alternatively, the change in the conformation of choline oxidase might occur during the freezing process in which the ice crystals are in the process of being formed (*55-57*), as previously proposed for phosvitin (*2*). Irrespective of whether the conformational change occurs in the frozen state or during the freezing process, such a structural change results in a conformation of choline oxidase that is not catalytically competent, as suggested by the lack of oxygen consumption with choline as substrate at pH 6 and 25 ^{o}C. The kinetic parameters determined at pH 7 for the partially reactivated enzyme previously frozen at low pH, with a lower k_{cat} and a similar K_m with respect to the native enzyme, provide independent evidence that *E*' is devoid of enzymatic activity, since these results are readily explained with the enzyme during steady state turnover at pH 7 existing as a mixture of fully active (*E*) and inactive forms (*E*').

Treatment of the inactive *E*' form of choline oxidase at pH values ≥6.5 and 25 ^{o}C results in the pH-dependent reactivation of ~30% of the enzyme, through a first-order kinetic process that occurs within the time frame of the oxygen electrode recordings (3 to 6 min). Evidence for this conclusion comes from the observed slow onsets of steady state when the *E*' form of the enzyme turned over with choline, as illustrated in the example of Figure 6.1. Consistent with reactivation of the enzyme being a first-order process, the apparent rate constant for reaching

steady state k_c was independent of the concentrations of both the enzyme and the choline substrate[6]. Furthermore, the k_{cat} and K_m values determined at pH 7, 8, and 9, for the enzyme previously frozen at pH 6 clearly suggest that only ~30% of the inactive enzyme could be reactivated within minutes through this pH-dependent process. A protein group with an apparent pK_a of ~7.3 that must be unprotonated is involved in the activation of the altered form of choline oxidase, as suggested by the pH-profile of the first-order rate constant k_c for reaching steady state during enzyme turnover. The slow pH-dependent reactivation of the inactive form of choline oxidase, along with the requirement of sub-freezing temperatures and low pH for the inactivation of the enzyme, strongly suggest that the reversible loss of enzymatic activity is due to the incorrect ionization of one or more protein groups that are not readily accessible to the solvent. In this respect, inactivation of the enzyme occurs irrespective of whether the enzyme is in the free form or in complex with glycine betaine, consistent with the ionizable group(s) responsible for the loss of catalytic activity not being in the active site of the enzyme. Similar reversible inactivations of enzymes being due to the incorrect ionization of protein groups have been previously proposed for ovoperoxidase (*10*), phosphofructose kinase (*11*), formyltetrahydrofolate synthetase (*9*), and leukocyte proteinase 3 (*12*).

The spectral perturbations induced in the UV-visible absorbance spectrum of choline oxidase upon freezing at low pH, with a prominent decrease in the intensity at 385 nm with respect to the native enzyme, resembled, although they were not identical to, those observed upon increasing pH with both the native and the active site variant $His_{466}Ala$ of the enzyme (*49*).

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[6] The length of the lag time required to reach steady state during enzymatic turnover of the altered choline oxidase was inversely proportional to the concentrations of both choline and enzyme. This is due to the rate of oxygen consumption at sub-saturating concentrations of choline increasing with both [choline] and [enzyme], and the rate constant for reaching steady state (k_c) being a first-order rate constant that is independent of both [choline] and [enzyme].

The spectral perturbations with those enzymes were interpreted as arising from the deprotonation of the N(3) locus of the enzyme-bound oxidized flavin (*58*). However, such a flavin position must be readily accessible to the solvent, as suggested by the observation that the spectral perturbations induced by pH in the native enzyme and the His_{466}Ala variant occur rapidly, ruling out a direct change in the ionization state of FAD as being responsible for the loss of enzymatic activity in choline oxidase. Nonetheless, the change in ionization state of the group that is responsible for the loss of enzymatic activity in choline oxidase has a direct effect on the spectral properties of the flavin, suggesting that such an ionizable group is either close to the flavin or linked to the flavin through a number of weak interactions, such as hydrogen bonds or salt bridges. Independent evidence that the conformational change induced in choline oxidase by freezing at low pH affects the microenvironment of the flavin comes from the both the 4 nm bathochromic shift of the band at 457 nm and the three-fold increase in flavin fluorescence, both consistent with an increased hydrophobicity of the flavin microenvironment in the inactive form of choline oxidase compared to the native enzyme (*58*).

At pH 6, the reactivation of the inactive form of choline oxidase is too slow to be seen within the time required for the acquisition of the oxygen traces, as suggested by the k_c value of ~3 x 10^{-3} s^{-1} estimated from the pH studies. Indeed, at this pH the enzyme did not show any oxygen consumption with 1 mM choline. However, prolonged incubation over 180 min of the inactive form of choline oxidase at pH 6 and 25 ^{o}C resulted in the slow and complete activation of the enzyme following a first-order process with a rate constant of activation k_{act} of ~4.7 x 10^{-4} s^{-1}. Evidence supporting the conclusion that the enzyme was slowly converted to the active form comes from the rate of oxygen consumption of ~4.5 s^{-1} with 1 mM choline determined after 180 min of incubation, which was similar to the rate of oxygen consumption of ~5 s^{-1} observed under

the same conditions with the native enzyme. The slow activation of choline oxidase was closely associated with a conformational change that reverted the enzyme to the native form, as suggested by the UV-visible absorbance spectral changes observed upon incubating the altered form of choline oxidase at pH 6 and 25 ^{o}C. Such spectral changes occurred according to a first-order kinetic pattern with a rate constant of ~3 x 10^{-4} s^{-1}, which is in reasonable agreement with the k_{act} value of ~5 x 10^{-4} s^{-1} determined for the rate of enzyme activation under the same conditions[7], consistent with the conformational change being closely associated with the gain in enzyme activity. Such a change in the conformation of the enzyme is likely localized, as suggested by the small and positive entropy of activation (TΔS) calculated at 25 ^{o}C from the experimental data, with a value of ~20 kJ mol^{-1}. For comparison, a larger TΔS value of ~90 kJ mol^{-1} was estimated at 25 ^{o}C for the pH-dependent reactivation of formyltetrahydrofolate synthetase, which was associated to a large conformational change accompanying the formation of the active tetramer from the inactive monomers of the enzyme (*9*).

In conclusion, the kinetic and spectroscopic data presented in this study are consistent with a localized conformational change in choline oxidase that is associated with the change in the ionization state of one or more protein groups, which is induced by freezing at low pH. The slow reactivation of the enzyme required elevated temperatures and was facilitated by high pH, suggesting that fairly drastic conditions are required to enable the enzyme "locked" in the inactive conformation to revert to the native form. Although the groups responsible for the

[7] The two rates reported here are not rigorously the same. However, some inaccuracies in the determination of the rate of activity increase for the altered form of enzyme can be envisioned due to the instability of the enzyme at 25 ^{o}C for 180 min. In this respect, the stability of the altered enzyme was established by following the decrease in activity of the native enzyme under the same conditions, with the assumption that the native and altered forms of choline oxidase share the same stability. Since the two forms of enzymes have different conformations, as established in this study, they might also have slightly different stability.

change in the protein conformation are not readily accessible to the solvent, and therefore are not likely to be in the enzyme active site, they have a direct effect on the microenvironment of the enzyme-bound flavin and the activity of the enzyme. While the basis for this phenomenon and its relationship to the mechanism of the reaction are not fully understood at a molecular level, this study shows that localized structural changes trap choline oxidase in a non-functional folded conformation that has reversibly lost the ability to catalyze the oxidation of choline.

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Chapter VII

GENERAL DISCUSSION

Choline oxidase from *Arthrobacter globiformis* is one of the rare flavin-dependent enzymes that are capable of catalyzing the four electron-oxidation of choline to glycine betaine within the same active site (*1-3*). Other than the interesting catalytic strategy, the enzyme was also studied due to its importance to the biotechnological and biomedical research. Early studies showed that glycine betaine was found to accumulate in the cytoplasm of cells in pathogenic bacteria (*4-13*) and transgenic plants (*14-19*) as a resistance for dehydration and plasmolysis in adverse hyperosmotic environments (*20, 21*). The understanding of the catalytic mechanism of choline oxidase gives insights into the biosynthesis pathway of glycine betaine found in human pathogens and economically relevant crop plants thereby provides foundations for drug development that can inhibit the stress resistance of pathogenic bacterial in human tissue or for bioengineering of transgenic plants with stress resistance to drought and salt stress. In this study, the chemical modification of choline oxidase with phenylhydrazine or tetranitromethane was performed with an attempt to identify the residues that are significant to the catalysis. Enzyme inactivation due to freezing in 20 mM sodium phosphate and 20 mM sodium pyrophosphate at pH 6 and -20 ^{o}C was also investigated as a measure of how storage conditions can affect the biochemical properties of choline oxidase.

Seven reagents were tested as inhibitors for choline oxidase, including 2-hydrazino-*N,N,N*-trimethyl-2-oxopethanaminium, allyltrimethylammonium, isonicotinic acid hydrazide, 3-dimethylamino-1-propyne, *trans*-2-phenylcyclopropylamine, phenylhydrazine, and tetranitromethane. The choice of reagents stemmed from previous studies, in which these compounds were reported to inhibit enzymes to significant extend and were successfully used in

modifying the active site of the enzyme. The resulted showed that only *trans*-2-phenylcyclopropylamine, phenylhydrazine, and tetranitromethane inactivated choline oxidase significantly in 20 mM Tris-Cl at pH 8 and 20 ^{o}C. *Trans*-2-phenylcyclopropylamine inactivated the enzyme with observed rates of 0.2-0.3 h^{-1} at 10-50 mM. This inhibitor may not be useful to modify the enzyme because large concentrations of inhibitor may cause unwanted alternation of enzyme structure or unspecific modification. Phenylhydrazine and tetranitromethane are better choices because they inactivated the enzyme with observed rate of 0.5-4.4 h^{-1} at 0.1-2.0 mM and 0.1-0.4 h^{-1} at 0.5-5.0 mM, respectively.

More than one modification sites may be obtained for choline oxidase when phenylhydrazine was used as the inhibitor, as suggested by kinetic and spectroscopic data from the chemical modification study. Phenylhydrazine inactivated choline oxidase irreversibly because there was no activity recovered when the inactive enzyme was isolated and incubated in 20 mM Tris-Cl at pH 8 and 20 ^{o}C. However, when the observed rate of inactivation was plotted as a function of the concentration of phenylhydrazine, a second-order inactivation rate suggested a reversible inhibitory process. A likely explanation for these results is that the observed rates of inactivation were still below the dissociation constant, K_I, of choline line oxidase with phenylhydrazine. The inhibitory mechanism of phenylhydrazine with choline oxidase occurred in the active site and involved one electron-transfer of the flavin cofactor, as suggested by the protective effect of glycine betaine and the formation of flavin semiquinone when the enzyme is incubated with phenylhydrazine at pH 8 and 15 ^{o}C in the presence of oxygen. Nevertheless, the generation of flavin semiquinone was not fully responsible for the inactivation of the enzyme because the percentage of the enzyme with the flavin semiquinone is not equivalent to the percentage of activity loss. When tryptic maps of glycine betaine-protected and unprotected

enzyme sample were compared, it appeared that phenylhydrazine modified enzyme at more than one site, i.e., more experiments are needed to improve the selectivity of the modification of essential residues in the active site of choline oxidase.

Choline oxidase can be inactivated when one or more tyosines in the active site of choline oxidase was modified with a nitro group. The evidence for this conclusion comes from the chemical modification study of choline oxidase with tetranitromethane. In that study, tetranitromethane inactivated choline oxidase irreversibly. The inactivation was both time and concentrations dependent. A saturation curve was observed when the observed rates of inactivation were plotted as a function of tetranitromethane, indicating the inactivation followed first-order kinetics. The maximal inactivation rate, k_{inact}, was 0.32 ± 0.01 h^{-1}, and the dissociation rate constant, K_I, was 0.66 ± 0.04 mM. These data suggested a reversible enzyme-inhibitor complex and an irreversible inactivation at saturating tetranitromethane concentrations. This is consistent with the inactivation of the enzyme by tetranitromethane being active-site directed, as suggested by the protective effect of glycine betaine on choline oxidase. UV-visible absorbance of both protected and unprotected enzyme sample showed the presence of nitrated tyrosines, but the unprotected sample had more intensity in absorbance equivalent to a difference of 5 tyrosines. Although choline oxidase has 11 tyrosines total, it is unlikely that there are 5 tyrosines present in its active site. The tryptic maps of protected and unprotected enzyme sample were also compared, the results suggested that there were various modification sites and no specific modification site was identified with mass spectrometry. More experiments and troubleshooting need to be performed in order to identify an essential tyrosyl residue, whose modification might be responsible for enzyme inactivation.

Choline oxidase can be inactivated due to a displacement of one or more protons and a localized conformation change in the active site, which can be induced with storage conditions. This conclusion is supported by the study of enzyme inactivation. In that study, the enzyme was inactivated after freezing in 20 mM sodium phosphate and 20 mM sodium pyrophosphate at pH 6 and -20 ^{o}C, as suggested by the complete loss of catalytic activity of the enzyme. Comparison of the spectroscopic properties between the inactive and the native forms of the enzyme at pH 6 and 15 ^{o}C showed that choline oxidase undergoes a localized conformation change upon freezing at pH 6 and -20 ^{o}C. Treatment at pH ≥6.5 and 25 ^{o}C can partially reactivate the enzyme, as suggested by the slow onset of steady state during enzymatic turnover. The conversion of inactive to active form of choline oxidase is a first-order rate constant that is independent of substrate and enzyme concentrations. However, the rate of conversion from inactive to active enzyme is pH dependent, yielding a pK_a value of 7.3 ± 0.1 for a group that needs to be unprotonated for the activation of the enzyme. The full activation of the enzyme can be observed spectroscopically at pH 6 and 25 ^{o}C in 3 h, with an increasing change in the absorbance at 385 nm. A temperature-dependence study of the enzyme at pH 6 and temperatures ≥20 ^{o}C yielded an $\Delta H^{\ddagger}$ of 112 ± 7 kJ mol^{-1} and an entropy of activation (T$\Delta S^{\ddagger}$) at 25 ^{o}C of 19 ± 2 kJ mol^{-1}. The data presented in this part of the study suggested that the inactivation of choline oxidase due to freezing at pH 6 and -20 ^{o}C involves a localized and reversible conformational change that is associated with a deprotonation of protein groups in the flavin microenvironment.

In summary, the results obtained in this study showed that choline oxidase can be inactivated by both chemical and physical means. Phenylhydrazine and tetranitromethane inactivated the enzyme irreversibly. Both compounds are active-site directed inhibitors. As for phenylhydrazine, it can be used as substrate for choline oxidase, and the inactivation with this

compound associated with the formation of flavin semiquinone in the presence of oxygen. However, the generation of flavin semiquinone is not fully responsible for enzyme inactivation. As for tetranitromethane, the inactivation associated with the increase in absorbance at 360 nm, where nitrated tyrosines absorbed. The modification sites in both studies were not identified due to nonselective reactivity of the compounds on the enzymes. Choline oxidase can also be inactivated by freezing in 20 mM sodium phosphate and 20 mM sodium pyrophosphate at pH 6 and -20 ^{o}C. The inactivation is reversible with pH $\geq$6.5 and at temperature $\geq$20 ^{o}C. The results suggested that enzyme reactivation required a group to be unprotonated, and the ionization of this group induced a localized conformational change that leads to enzyme inactivation. By studying enzyme inactivation due to chemical and physical means, we have gained insights into the biochemical properties of choline oxidase, thereby adding useful information onto the understanding of this enzyme, such as how enzyme function can be affected significantly by the modification at the active site or the displacement of a proton and a slight conformational change in the microenvironment of the flavin cofactor. Consequently, the findings reported in this thesis may provide insights into the importance of the rigorous organization in the active site of choline oxidase; slight alteration of this organization can result in the complete loss of biological function of the enzyme.

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