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Role of Asparagine 510 in the Relative Timing of Substrate Bond Cleavages in the Reaction Catalyzed by Choline Oxidase†

Kunchala Rungsrisuriyachai‡ and Giovanni Gadda*,‡,§,∥

‡*Departments of Chemistry*, §*Biology, and* ∥*The Center for Biotechnology and Drug Design, Georgia State University, Atlanta, Georgia 30302-4098*

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ABSTRACT: The flavoprotein choline oxidase catalyzes the oxidation of choline to glycine betaine with transient formation of an aldehyde intermediate and molecular oxygen as final electron acceptor. The enzyme has been grouped in the glucose−methanol−choline oxidoreductase enzyme superfamily, which shares a highly conserved His-Asn catalytic pair in the active site. In this study, the conserved asparagine residue at position 510 in choline oxidase was replaced with alanine, aspartate, histidine, or leucine by site-directed mutagenesis, and the resulting mutant enzymes were purified and characterized in their biochemical and mechanistic properties. All of the substitutions resulted in low incorporation of FAD into the protein. The Asn510Asp enzyme was not catalytically active with choline and had 75% of the flavin associated noncovalently. The most notable changes in the catalytic parameters with respect to wild-type choline oxidase were seen in the Asn510Ala enzyme, with decreases of 4300-fold in the $k_{cat}/K_{choline}$, 600-fold in the k_{red}, 660-fold in the k_{cat}, and 50-fold in the k_{cat}/K_{oxygen} values. Smaller, but nonetheless similar, changes were seen also in the Asn510His enzyme. Both the K_d and K_m values for choline changed ≤7-fold. These data are consistent with Asn510 participating in both the reductive and oxidative half-reactions but having a minimal role in substrate binding. Substrate, solvent, and multiple kinetic isotope effects on the k_{red} values indicated that the substitution of Asn510 with alanine, but not with histidine, resulted in a change from stepwise to concerted mechanisms for the cleavages of the OH and CH bonds of choline catalyzed by the enzyme.

The flavoenzyme choline oxidase (E.C. 1.1.3.17; choline−oxygen 1-oxidoreductase) catalyzes the two-step oxidation of choline to glycine betaine with the formation of an aldehyde as reaction intermediate (Scheme 1). The study of the biophysical and mechanistic properties of choline oxidase is interesting for medical and biotechnological applications because accumulation of the biocompatible solute glycine betaine in many pathogens and plants enables their stress resistance toward hyperosmotic environments (*1*, *2*). Mechanistic studies on choline oxidase have been carried out by using pH, kinetic isotope, and temperature effects (*3*−*7*), as well as site-directed mutagenesis on a number of active site residues (*8*−*12*), mainly to address the mechanism of alcohol oxidation. Catalysis is initiated with the deprotonation of the hydroxyl group of choline to yield a zwitterionic alkoxide species, which is stabilized in the enzyme active site through electrostatic and hydrogen-bonding interactions with the charged side chains of His351, His466, and Glu312 (*9*−*12*). A hydride ion subsequently transfers from the alkoxide α-carbon of the activated substrate to the flavin N(5) atom (*4*). After the reduced flavin reacts with oxygen to yield hydrogen peroxide and regenerate oxidized flavin, the enzyme undergoes a second oxidation step that involves the hydrated form of the aldehyde to generate glycine betaine and hydrogen peroxide (*13*).

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*To whom correspondence should be addressed at the Department of Chemistry, Georgia State University, P.O. Box 4098, Atlanta, GA 30302-4098. Phone: (404) 413-5537. Fax: (404) 413-5505. E-mail: ggadda@gsu.edu.

Choline oxidase has been grouped in the glucose−methanol−choline (GMC) oxidoreductase enzyme superfamily (*14*, *15*), which comprises choline dehydrogenase (*16*), cellobiose dehydrogenase (*17*), glucose oxidase (*18*), pyranose 2-oxidase (*19*, *20*), methanol oxidase, and cholesterol oxidase (*21*). The active sites of representative enzymes within the superfamily reveal the presence of a conserved His-Asn pair, except for glucose oxidase where the asparagine residue is replaced with histidine (*20*). Site-directed mutagenesis studies on the role of the conserved asparagine residues are limited to cholesterol oxidase and cellobiose dehydrogenase. In cholesterol oxidase, Asn485 has been shown to hydrogen bond to the flavin π-system, thereby modulating the electrostatic potential of the flavin to enhance the oxidation reaction (*22*). In cellobiose dehydrogenase, Asn732 has been implicated in substrate binding (*23*). In choline oxidase, the side chain of Asn510 interacts with the imidazole side chains of His351 (3.0 Å) and His466 (4.0 Å), and it is 4.7 Å from both the flavin O(2) and N(3) atoms (Figure 1). In a recent study, His351 was shown to participate in substrate binding and in the hydride ion transfer reaction, as suggested by the effect on the K_d and k_{red} values with choline as substrate for the mutant enzyme containing alanine at position 351 (*12*). Site-directed mutagenesis studies allowed the authors to propose multiple roles for His466 in modulating the electrophilicity of the flavin and the polarity of the active site and contributing to the stabilization of the transient alkoxide species that is formed in the oxidation of choline (*9*).

In this study, we assess the mechanistic role of Asn510 in choline oxidase using site-directed mutagenesis and biochemical, steady-state, and rapid kinetics techniques. Four mutant variants

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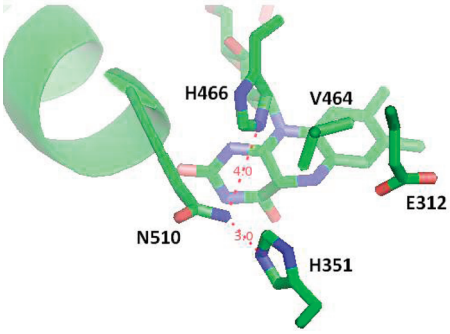
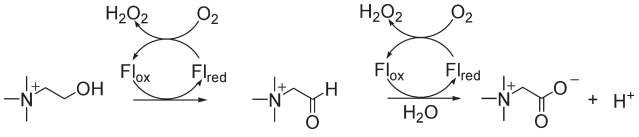


FIGURE 1: The active site of wild-type choline oxidase with selected amino acids at a resolution of 1.86 Å (PDB 2jbv). Dashed, red lines represent interactions of the side chain of Asn510 with the side chains of His351 and His466.

Scheme 1: Reaction Catalyzed by Choline Oxidase



where Asn510 is replaced with alanine, leucine, aspartate, and histidine were prepared using site-directed mutagenesis, and the resulting proteins were purified to high levels and investigated in their biochemical and mechanistic properties. The results of the spectroscopic and mechanistic investigations presented here establish the importance of Asn510 in both the reductive and oxidative half-reactions catalyzed by choline oxidase. Interestingly, the relative timing of cleavages of the substrate OH and CH bonds in the mutant enzymes differed among mutated enzymes, depending upon which substitution is used to replace Asn510.

EXPERIMENTAL PROCEDURES

Materials. *Escherichia coli* strain Rosetta(DE3)pLysS was from Novagen (Madison, WI). DNase was from Roche. The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The QIAprep Spin Miniprep kit was from Qiagen (Valencia, CA). Oligonucleotides used for sequencing of the mutant gene were custom synthesized by Sigma Genosys (Woodland, TX). Bovine serum albumin, chloramphenicol, tetracycline, DMSO, isopropyl β-D-thiogalactopyranoside (IPTG), phenylmethanesulfonyl fluoride (PMSF), lysozyme, sodium hydrosulfite, betaine aldehyde, glycine betaine, and Luria−Bertani agar and broth were from Sigma (St. Louis, MO). EDTA was from Fisher. Choline chloride and ampicillin were from ICN Pharmaceutical Inc. 1,2-[$^2H_4$]Choline bromide (98%) and sodium deuterioxide (99%) were from Isotec Inc. (Miamisburg, OH). All other reagents were of the highest purity commercially available.

Site-Directed Mutagenesis. Mutant genes for the choline oxidase variants Asn510Ala,[1] Asn510Asp, Asn510His, and Asn510Leu were prepared using the pET/codAmg1 plasmid for the wild-type enzyme as a template (*24*) and forward and reverse oligonucleotides as primers for site-directed mutagenesis. A QuikChange kit was used to prepare the mutant enzymes, and the experiment was performed by following the manufacturer's instructions in the presence of 2% DMSO. The resulting mutant genes were sequenced at the DNA Core Facility at Georgia State University using an Applied Biosystems Big Dye kit on an Applied Biosystems model ABI 377 DNA sequencer. Sequencing confirmed the presence of the mutant genes in the correct orientation.

Expression and Purification of Choline Oxidase Variants. Permanent stocks of *E. coli* Rosetta(DE3)pLysS cells harboring plasmids pET/*codA*mg1-Asn510Ala, -Asn510Asp, -Asn510His, or -Asn510Leu were used to inoculate 4.5 L of Luria−Bertani broth medium containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. The liquid cultures were grown for 4−6 h at 37 °C, before inducing protein expression with 0.1 mM IPTG for 20 h at 20 °C. The variant enzymes were expressed and purified to homogeneity using the same procedure described previously for the purification of the wild-type enzyme (*24*).

Spectrophotometric Studies. The extinction coefficients of the mutant enzymes were determined in Tris-HCl, pH 8.0, after denaturation of the enzymes by treatment with 4 M urea at 40 °C for 30 min, based upon the ε_{450} value of 11.3 mM^{-1} cm^{-1} for free FAD (*25*). To determine the amount of covalently bound flavin, the purified enzymes were incubated on ice for 30 min after the addition of 10% trichloroacetic acid, followed by removal of precipitated protein by centrifugation. The UV−visible absorbance spectra of the supernatants were recorded again to check the presence of unbound FAD. All UV−visible absorbance spectra were recorded using an Agilent Technologies diode-array model HP 8453 spectrophotometer.

Steady-State Kinetics. The enzymatic activity of choline oxidase mutants was measured by the method of initial rates as described for the wild-type enzyme (*7*, *24*) using a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.). The steady-state kinetic parameters of the Asn510Ala and Asn510His enzymes were determined at varying concentrations of choline between 1 and 10 mM and oxygen between 0.05 and 0.75 mM in 50 mM sodium pyrophosphate, pH 10.0 and 25 °C. Each reaction mixture was equilibrated at the desired oxygen concentration by sparging the appropriate O_2/N_2 gas mixture for a minimum of 15 min before the reaction was started with the addition of the enzyme.

Reductive Half-Reactions. Reductive half-reactions were carried out by using a Hi-Tech SF-61 stopped-flow spectrophotometer thermostated at 25 °C, pH or pD 10.0. The rate of flavin reduction was measured by monitoring the decrease in absorbance at 454 nm that results from the anaerobic mixing of choline oxidase with choline or betaine aldehyde, as previously described for the wild-type enzyme (*3*). Glucose (5 mM) and glucose oxidase (0.5 μM) were added to the substrate and enzyme solutions to scavenge possible trace amounts of oxygen. The mutant enzymes Asn510Ala and Asn510His were mixed anaerobically with an equal volume of substrate, obtaining reaction mixtures with 20 μM enzyme and 0.5−25 mM choline or 1,2-[$^2H_4$]choline or 20 μM enzyme and 0.1−5 mM betaine aldehyde. For the determination of solvent isotope effects, buffers were prepared using 99.9% deuterium oxide by adjusting the pD value with NaOD. The pD values were determined by adding 0.4 to the pH electrode readings (*26*). For each concentration of the substrates, the rate constants for flavin reduction were recorded in triplicate, with measurements usually differing by ≤5%. Solvent viscosity effects were measured in the presence of 0.0211 g/mL PEG-6000 as viscosigen, in both the tonometer containing the enzyme and the syringes containing the organic substrates. The resulting relative viscosity at 25 °C was 1.26,

[1]Abbreviations: Asn510Ala, Asn510His, Asn510Leu, and Asn510Asp enzymes, choline oxidase variants with asparagine at position 510 replaced with alanine, histidine, leucine, and aspartate.

Table 1: Apparent Steady-State Kinetic Parameters for Choline Oxidase Variants Substituted at Asn510[a]

	wild type[b]	Asn510His	Asn510Ala	Asn510Leu	Asn510Asp
$^{app}k_{cat}$, s^{-1}	13.4 ± 0.5	0.51 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	nd[c]
$^{app}K_{choline}$, mM	0.6 ± 0.1	60 ± 4	155 ± 34	213 ± 22	nd
$^{app}(k_{cat}/K_{choline})$, M^{-1} s^{-1}	22000 ± 3760	8.5 ± 0.6	0.4 ± 0.1	0.09 ± 0.01	nd

[a]Initial rates determined as oxygen consumption with choline as substrate at fixed atmospheric [oxygen] in 50 mM potassium phosphate, pH 7.0 and 25 °C. [b]From ref *11*. [c]Not determined.

which is slightly above the value of 1.23 representing a 100% solution of D_2O (*27*, *28*).

Data Analysis. Kinetic data were fit with the KaleidaGraph (Synergy Software, Reading, PA) and the Enzfitter (Biosoft, Cambridge, U.K.) softwares. The steady-state kinetic parameters at varying concentrations of both choline and oxygen were determined by fitting the initial rates to eq 1, which describes a steady-state kinetic mechanism with formation of a ternary complex. Here, K_a and K_b are the Michaelis constants for choline (*A*) and oxygen (*B*), respectively, and k_{cat} is the turnover number of the enzyme (*e*) at saturating concentrations of both substrates. For kinetic isotope effects with choline as substrate, data obtained were divided into two sets, one with unlabeled substrate or solvent and one with isotopically labeled substrate or solvent, and kinetic isotope effects were determined by taking the ratio of the kinetic parameters obtained with normal substrate or solvent to that obtained with labeled substrate or solvent. Stopped-flow traces were fit to eq 2, which describes a single exponential process where k_{obs} is the observed first-order rate constant for flavin reduction, *A* is the value of absorbance at the specific wavelength of interest at time *t*, *B* is the amplitude of the absorbance change, and *C* is an offset value that accounts for the nonzero absorbance value at infinite time. Kinetic parameters for the reductive half-reactions were determined by using eq 3, where k_{obs} is the observed first-order rate constant for the reduction of the enzyme-bound flavin at any given concentration of substrate, k_{red} is the limiting first-order rate constant for flavin reduction at saturated substrate concentration, and K_d is the macroscopic dissociation constant for binding of the substrate to the enzyme.

$$\frac{v}{e} = \frac{k_{cat}AB}{K_aB + K_bA + AB + K_{ia}K_b} \quad (1)$$

$$A = B\exp(-k_{obs}t) + C \quad (2)$$

$$k_{obs} = \frac{k_{red}A}{K_d + A} \quad (3)$$

RESULTS

Expression and Purification of Asn510 Variants of Choline Oxidase. The mutant proteins containing alanine, aspartate, histidine, or leucine at position 510 were expressed and purified at pH 8.0 to high levels as judged by SDS−PAGE using the same protocol that was devised for the wild-type enzyme (*24*). Glycerol (10%) was present in all of the buffered solutions throughout the purification process to increase the stability of the enzymes (*29*). With the exception of the Asn510His enzyme, which stabilized aerobically the anionic form of the flavin semiquinone as previously reported for the wild-type enzyme (*7*), the Asn510Ala, Asn510Asp, and Asn510Leu enzymes were in the oxidized state throughout the purification process at pH 8.0.

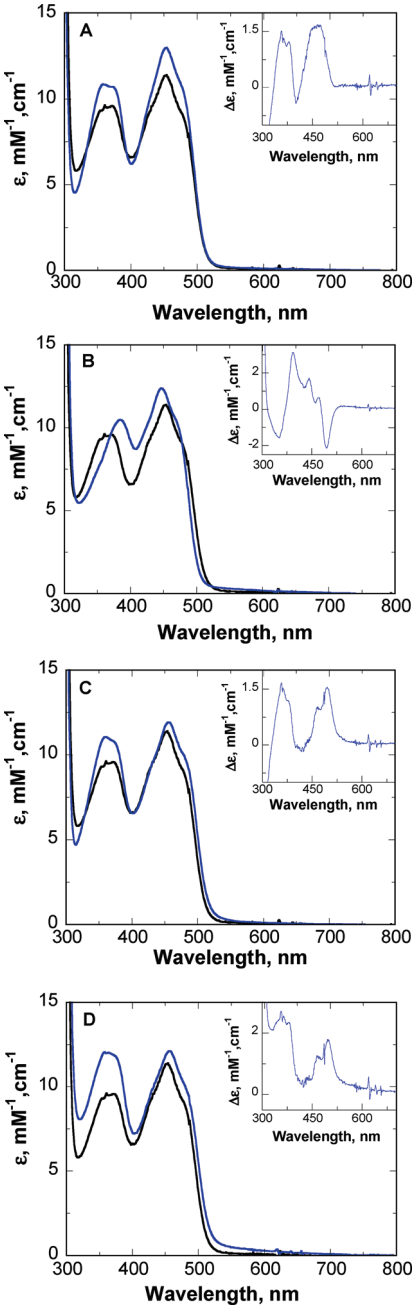


FIGURE 2: Comparison of the UV−visible absorbance spectra of the oxidized wild-type (black curve) and Asn510 mutants (blue curves) in 20 mM Tris-HCl, pH 8.0: (A) Asn510Ala, (B) Asn510Asp, (C) Asn510His, and (D) Asn510Leu enzymes. The insets show the difference absorbance spectra of the Asn510 mutant enzymes minus the wild-type enzyme.

The Asn510Asp enzyme showed no oxygen consumption at concentrations as high as 20 μM and up to 0.6 M choline at pH 7.0 and 25 °C (for comparison, the wild-type enzyme typically shows maximal velocities in the ~90 nmol of O_2 mL^{-1} min^{-1}

Table 2: Comparison of Spectral Parameters for Choline Oxidase Variants Substituted at Asn510 at pH 8.0

	wild type[a]	Asn510His	Asn510Ala	Asn510Leu	Asn510Asp
UV−visible absorbance (λ_{max}, nm)	366, 452	360, 456	358, 454	364, 455	384, 446
ε (mM^{-1} cm^{-1})	9.8, 11.4	11.1, 11.8	10.6, 13.0	11.5, 12.1	10.5, 12.4
stoichiometry (no. of FAD/protein)	0.9 ± 0.1	0.16 ± 0.01	0.26 ± 0.01	0.22 ± 0.01	0.18 ± 0.01
% of FAD noncovalently bound	0	0	0	0	75

[a]From ref *7*.

Table 3: Comparison of Kinetic Parameters of Asn510Ala and Asn510His Enzymes with Wild-Type Choline Oxidase[a]

	kinetic parameter	wild type[b]	Asn510His	Asn510Ala
choline	k_{cat}, s^{-1}	60 ± 1	3.4 ± 0.2	0.090 ± 0.003
	$K_{choline}$, mM	0.25 ± 0.01	0.48 ± 0.08	1.7 ± 0.2
	K_{oxygen}, μM	690 ± 30	535 ± 60	55 ± 12
	$k_{cat}/K_{choline}$, M^{-1} s^{-1}	237000 ± 9000	7100 ± 1250	55 ± 6
	k_{cat}/K_{oxygen}, M^{-1} s^{-1}	86400 ± 3600	6360 ± 805	1700 ± 370
	K_{ia}, mM	0.14 ± 0.01	0.23 ± 0.06	3.45 ± 1.37
	k_{red}, s^{-1}	93 ± 1	4.3 ± 0.2	0.13 ± 0.01
	K_d, mM	0.29 ± 0.01	1.2 ± 0.2	0.95 ± 0.06
betaine aldehyde	k_{red}, s^{-1}	135 ± 4	17.0 ± 0.6	0.88 ± 0.01
	K_d, mM	0.45 ± 0.03	1.0 ± 0.1	5.9 ± 0.2

[a]Steady-state parameters were measured at varying concentrations of both choline and oxygen in 50 mM sodium pyrophosphate, pH 10, at 25 °C. [b]From ref *3*.

when assayed at a concentration of 0.1 μM). The apparent steady-state kinetic parameters for all of the other mutant enzymes were determined with choline at atmospheric oxygen concentration, yielding apparent turnover numbers ($^{app}k_{cat}$) at least 25-fold lower than that of the wild-type enzyme and $^{app}K_{choline}$ values that were at least 100-fold higher than that of the wild-type enzyme (Table 1). Thus, Asn510 is an important residue for catalysis in the active site of choline oxidase.

Flavin Content. The UV−visible absorbance spectra of the Asn510 mutant enzymes as purified had maxima in the 360 and 450 nm regions[2], as expected for flavoproteins with the flavin in the oxidized state (Figure 2). The Asn510Asp enzyme showed the most dramatic changes with a hypsochromic shift of 6 nm of the low energy band of the flavin and a 22 nm bathochromic shift of the high energy band of the flavin with respect to the wild-type enzyme (Figure 2). The Asn510Asp enzyme also had 75% of the total flavin noncovalently associated with the protein, as established through acid denaturation with 10% trichloroacetic acid followed by centrifugation to remove denatured protein. In contrast, the Asn510His, Asn510Ala, and Asn510Leu enzymes showed small bathochromic shifts (≤4 nm) of the low energy flavin band and small hypsochromic shifts (≤8 nm) of the high energy flavin band (Figure 2). These mutant enzymes had the flavin covalently linked to the protein, as for the case of the wild-type enzyme (*7*, *24*). With all of the mutant enzymes, a flavin to protein ratio between 0.16 and 0.26 was determined, which was significantly lower than the value of 0.90 determined for the wild-type enzyme (Table 2). Collectively, these results suggest that substitution of Asn510 with other amino acids results in low incorporation of the flavin in the protein, an altered protein microenvironment around the flavin, and, for the case of the Asp510 enzyme, impairment of protein flavinylation.

[2]The Asn510His enzyme required extensive dialysis at pH 6.0 and 4 °C for the conversion of the anionic flavosemiquinone to the oxidized state, as for the case of the wild-type enzyme (*7*, *24*).

Kinetic Properties. A detailed kinetic characterization was carried out for the Asn510His and Asn510Ala enzymes by using steady-state and rapid kinetics approaches but not for the Asn510Leu and Asn510Asp enzymes for which enzymatic activity was too low to obtain meaningful data. The steady-state kinetic parameters were determined by monitoring the initial rates of oxygen consumption at varying concentrations of both choline and oxygen at pH 10.0 and 25 °C. The choice of pH 10.0 was dictated by previous investigations on the wild-type enzyme and various mutant forms showing that the k_{cat} and the $k_{cat}/K_{choline}$ values of choline oxidase are pH-independent at high pH (*3*, *4*, *6*, *7*, *9*, *11*−*13*). With both the Asn510His and Asn510Ala enzymes, the best fit of the data was observed with a sequential steady-state kinetic mechanism as seen also in the wild-type enzyme (*3*, *5*, *6*). The most notable changes in the kinetic parameters with respect to the wild-type enzyme were seen in the Asn510Ala enzyme, with decreases of 4300-fold in the $k_{cat}/K_{choline,}$ 660-fold in the k_{cat}, and 50-fold in the k_{cat}/K_{oxygen} values (Table 3). For comparison, the Asn510His enzyme showed smaller decreases of 30-fold in the $k_{cat}/K_{choline}$ and ∼15-fold in both the k_{cat} and k_{cat}/K_{oxygen} values (Table 3). These data suggest that replacing Asn510 with alanine or histidine negatively affects both the reductive and oxidative half-reactions catalyzed by choline oxidase.

The reductive half-reactions of the Asn510His and Asn510Ala enzymes were also investigated in a stopped-flow spectrophotometer by mixing anaerobically the enzyme with choline or betaine aldehyde at pH 10.0 and 25 °C. The time-resolved changes in the absorbance at ∼455 nm were best fit to a single exponential process, as illustrated in the example of Figure 3. Full reduction to the anionic hydroquinone species of the flavin was observed (Figure 3). The observed rate constants for flavin reduction showed hyperbolic dependence on the concentration of choline or betaine aldehyde, allowing the determination of the limiting rate constants for flavin reduction (k_{red}) and the equilibrium constants for substrate binding (K_d). As illustrated in Table 3, significant decreases in the k_{red} values with respect to the

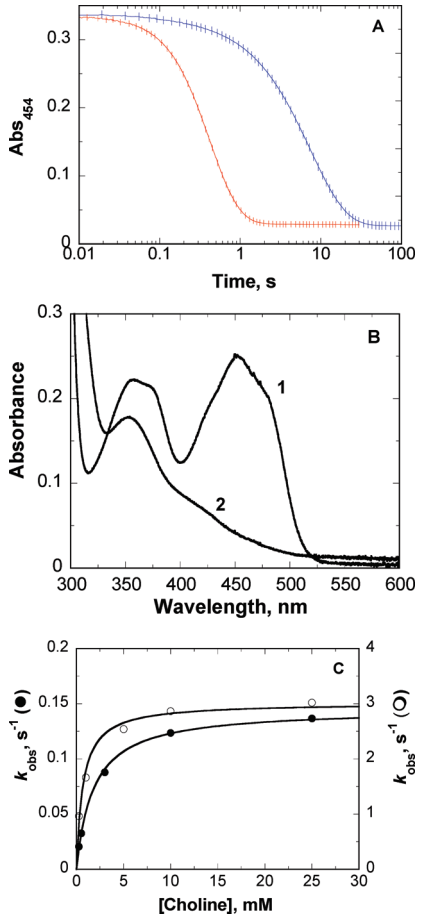


FIGURE 3: Anaerobic substrate reductions of the Asn510Ala and Asn510His enzymes with choline as substrate. (A) Stopped-flow traces obtained upon mixing anaerobically the Asn510Ala (blue) and Asn510His (red) enzymes with 25 mM choline in 50 mM sodium pyrophosphate buffer, pH 10.0, at 25 °C. The curves represent the fit of the data with eq 2. Time indicated is after the end of flow, i.e., 2.2 ms, and one experimental point in every 10 is shown (vertical lines). (B) UV−visible absorbance spectra of the oxidized (curve 1) and reduced (curve 2) species of the Asn510Ala enzyme after mixing anaerobically the oxidized enzyme with buffer and 25 mM choline in 50 mM sodium pyrophosphate buffer, pH 10.0, at 25 °C. (C) The observed rate constants of anaerobic flavin reduction (k_{obs}) of the Asn510Ala (●) and Asn510His (○) enzymes as a function of the concentrations of choline. Data were fit to eq 3.

wild-type enzyme were seen in both enzymes with both substrates. The K_d values for choline and betaine aldehyde were ≤13-fold larger than the value of wild-type choline oxidase, consistent with replacement of Asn510 with alanine or histidine having only a minor effect on substrate binding.

Kinetic Isotope Effects. The relative timing for the cleavage of the OH and CH bonds of choline in the reactions catalyzed by the Asn510His and Asn510Ala enzymes was investigated with substrate, solvent, and multiple kinetic isotope effects on the reductive half-reaction in a stopped-flow spectrophotometer at pL 10.0 and 25 °C. Substitution of choline with 1,2-[$^2H_4$]choline yielded $^{D}k_{red}$ values >4 with both the Asn510His and Asn510Ala enzymes (Table 4), indicating that the cleavage of the CH bond of choline is at least partially rate limiting in the reductive half-reaction. Substitution of water with deuterium oxide resulted in $^{D_2O}k_{red}$ values >1.3 with both enzymes (Table 4), suggesting that the cleavage of the OH bond of choline is also at least partially rate limiting in the reductive half-reaction. These data are significantly different from those with the wild-type form of choline oxidase, for which the $^{D}k_{red}$ values are large and the $^{D_2O}k_{red}$ values are unity (*3*).

Table 4: Substrate and Solvent Kinetic Isotope Effects with Choline as Substrate[a]

parameter	wild type[b]	Asn510His	Asn510Ala
$^{D}k_{red}$	8.9 ± 0.2	4.2 ± 0.1	5.5 ± 1.2
$^{D}(k_{red})_{D_2O}$	8.7 ± 0.2	1.9 ± 0.1	7.2 ± 0.4
$^{D_2O}k_{red}$	0.99 ± 0.02	2.6 ± 0.1	1.28 ± 0.04
$^{D_2O}(k_{red})_{D}$	0.94 ± 0.03	1.23 ± 0.04	1.7 ± 0.4
$^{D,D_2O}k_{red}$	8.4 ± 0.2	5.2 ± 0.2	9.2 ± 0.6

[a]Conditions: 50 mM sodium pyrophosphate, pH or pD 10, at 25 °C. [b]From ref *3*.

With the Asn510His enzyme, a significant decrease in the magnitude of the substrate kinetic isotope effect was observed upon substituting water with deuterium oxide (cf. $^{D}(k_{red})_{D_2O}$ and $^{D}k_{red}$ values in Table 4). Analogously, the magnitude of the solvent kinetic isotope effect also decreased when 1,2-[$^2H_4$]choline was used instead of choline (cf. $^{D_2O}(k_{red})_{D}$ and $^{D_2O}k_{red}$ values in Table 4). Finally, multiple kinetic isotope effects were smaller than the product of the individual substrate and solvent kinetic isotope effects (cf. $^{D,D_2O}k_{red}$ and the product of the $^{D}k_{red}$ and $^{D_2O}k_{red}$ values in Table 4).

With the Asn510Ala enzyme, the substrate kinetic isotope effect increased upon substituting water with deuterium oxide (cf. $^{D}(k_{red})_{D_2O}$ and $^{D}k_{red}$ values in Table 4), as did the solvent kinetic isotope effect when 1,2-[$^2H_4$]choline was used instead of choline (cf. $^{D_2O}(k_{red})_{D}$ and $^{D_2O}k_{red}$ values in Table 4). Furthermore, the $^{D,D_2O}k_{red}$ value was slightly larger than the product of the $^{D}k_{red}$ and $^{D_2O}k_{red}$ values.

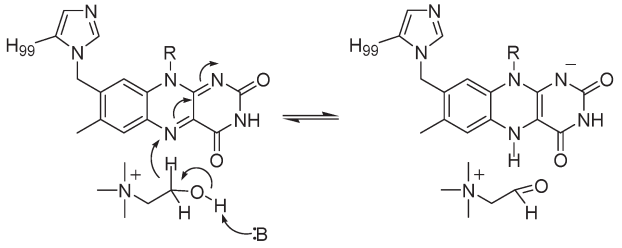
To establish whether the observed solvent kinetic isotope effects originated from the cleavage of the OH bond of choline rather than being due to an increased viscosity of D_2O with respect to H_2O, the effects of solvent viscosity on the reductive half-reactions of the Asn510His and Asn510Ala enzymes were investigated. The experiment was performed at pH 10.0 and 25 °C in solutions containing 0.0211 g/mL PEG-6000, which provides a relative solvent viscosity equivalent to a 100% solution of D_2O. In both enzymes, similar k_{red} values were observed in the presence and absence of viscosigen (i.e., 4.3 ± 0.2 s^{-1} versus 4.4 ± 0.2 s^{-1} in the Asn510His enzyme and 0.13 ± 0.01 s^{-1} versus 0.13 ± 0.01 s^{-1} in the Asn510Ala enzyme). Similarly, the K_d values were not affected by the increased relative viscosity of the solvent (data not shown). These data indicate that the solvent kinetic isotope effects determined in D_2O were directly associated with the cleavage of the substrate OH bond in the reaction catalyzed by Asn510His and Asn510Ala enzymes.

DISCUSSION

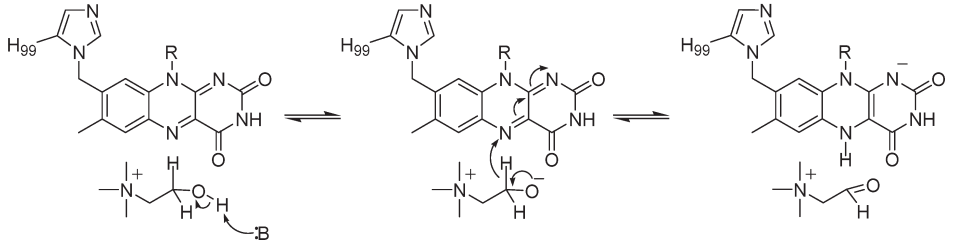
The variants of choline oxidase in which the conserved residue Asn510 was replaced with alanine, aspartate, histidine, or leucine were prepared in this study to investigate the role of this residue in enzyme catalysis. The mutant enzymes maintained a number of biophysical and kinetic properties that are in common with the wild-type form of choline oxidase. The Asn510Ala, Asn510His, and Asn510Leu enzymes contained FAD covalently linked to the protein moiety, as does the wild-type enzyme. The Asn510Ala and Asn510His enzymes displayed a sequential steady-state kinetic mechanism as does the wild-type enzyme and had K_m and K_d values for choline that differed by less than 7 times from the values of the wild-type enzyme. Moreover, large substrate

Scheme 2: Relative Timing for Cleavages of OH and CH Bonds of Choline in Reactions Catalyzed by Ans510His and Asn510Ala Enzymes

Asn510Ala enzyme:



Asn510His enzyme:



kinetic isotope effects with values ≥4 were determined in the reductive half-reactions of the Asn510Ala, Asn510His, and wild-type enzymes. Finally, the small perturbations of the UV−visible absorbance spectra of all of the four mutant enzymes from the features observed in the wild-type enzyme can be rationalized with the effects that a substituted protein microenvironment have on the spectroscopic properties of the enzyme-bound flavin (see below), further suggesting that the enzymes maintain an overall fold that is similar to that of the wild-type enzyme. These observations allowed conclusions on the role of Asn510 in the reaction catalyzed by choline oxidase to be drawn from the comparison of the biophysical, kinetic, and mechanistic properties of the enzyme variants containing amino acids other than asparagine at position 510 with those of the wild-type enzyme.

The active site residue Asn510 is important for the oxidation of both choline and the reduced flavin, but plays a minor role in the binding of the alcohol substrate. Evidence supporting this conclusion comes from the comparison of the steady-state and rapid reaction kinetic data of the Asn510His and Asn510Ala enzymes with the wild-type form of choline oxidase. Both the bimolecular rate constant for the reductive half-reactions ($k_{cat}/K_{choline}$) and the limiting rate constant for anaerobic flavin reduction (k_{red}) were decreased by >600-fold and >30-fold upon replacing Asn510 with alanine and histidine. The second-order rate constant for oxygen capture on the enzyme (k_{cat}/K_{oxygen}) decreased 15-fold in the Asn510His enzyme and 50-fold in the Asn510Ala enzyme. Finally, the equilibrium constant for substrate binding (K_d) increased by ≤6-fold when the mutant enzymes were compared to wild-type choline oxidase. Small increases in the K_m values were reported for mutant variants of another member of the glucose−methanol−choline oxidoreductase superfamily, cellobiose dehydrogenase, where Asn732 was replaced with alanine (no changes), histidine or glutamine (2.5-fold), aspartate (10-fold), and glutamate (14-fold). In that case the authors concluded that the main role of Asn732 is in the binding of the substrate (*23*).

The overall turnover in the Asn510 variants with choline as substrate is limited primarily by the chemical step of hydride transfer from the alcohol substrate to enzyme-bound flavin, with contribution of the second hydride transfer involving the aldehyde intermediate and the flavin. This conclusion is supported by steady-state and rapid kinetic data and the use of eq 4, where $k_{red(CH)}$ and $k_{red(BA)}$ represent the limiting rate constants for the hydride transfer reactions between choline and betaine aldehyde and the enzyme-bound flavin. The k_{cat} values calculated by using eq 4 were 0.1 s^{-1} and 3.4 s^{-1} for the Asn510Ala and Asn510His enzymes, in excellent agreement with the experimentally measured k_{cat} values of 0.09 s^{-1} and 3.4 s^{-1} with choline as substrate. The overall turnover being limited by both hydride transfer reactions implies that choline is oxidized to glycine betaine in the reaction catalyzed by the Asn510 mutant enzymes, as for the case previously reported for the wild-type enzyme (*3*).

$$k_{cat} = \frac{k_{red(CH)}k_{red(BA)}}{k_{red(CH)} + k_{red(BA)}} \quad (4)$$

In the reductive half-reaction, replacement of Asn510 with histidine or alanine decreases the rate of cleavage of the substrate OH bond catalyzed by choline oxidase. This conclusion is supported by the solvent kinetic isotope effects on the limiting rate constant for anaerobic flavin reduction at saturating concentration of choline, $^{D_2O}k_{red}$, being significantly larger than unity, with values of 2.6 and 1.3 in the Asn510His and Asn510Ala enzymes. This is not the case for the wild-type form of choline oxidase, for which it was shown that the cleavage of the substrate OH bond is significantly faster than the subsequent cleavage of the CH bond, as indicated by a $^{D_2O}(k_{red})$ value of 0.99 (*3*). Slower rates of hydroxyl proton abstraction with respect to the wild-type enzyme were recently reported for other variants of choline oxidase where Val464 is replaced with threonine or alanine, where the $^{D_2O}k_{red}$ values were >4 (*8*). Interestingly, the side chains of Val464 and Asn510 are both in spatial proximity of His466 (i.e., ≤4 Å), a residue that in choline oxidase has been implicated in the electrostatic stabilization of the transient alkoxide species that originates from the cleavage of the substrate OH bond (*9*). Thus, it is likely that the much slower rate of hydroxyl proton abstraction in these mutant enzymes is due to a nonoptimal orientation of the side chain of His466 with respect to

either the base that abstracts the substrate proton, the alcohol substrate, or both. In support of a disrupted preorganization in the enzyme−substrate complexes of the mutant enzymes is the observation of a substrate kinetic isotope effect that is significantly lower than the value of 9 that is seen in the wild-type enzyme (*3*).

The relative timing for cleavages of the substrate OH and CH bonds is affected upon replacing Asn510 with alanine, but not when Asn510 is substituted with histidine (Scheme 2). Evidence for this conclusion comes from the comparison of the multiple kinetic isotope effects determined on the limiting rate constants for flavin reduction at saturating concentrations of choline for the Asn510Ala, Asn510His, and wild-type enzymes. With the Asn510Ala enzyme, both the substrate and solvent kinetic isotope effects increased upon slowing down the cleavage of the other bond by replacing water with D_2O and choline with 1,2-[$^2H_4$]choline, respectively. This immediately rules out a stepwise mechanism for cleavage of the substrate OH and CH bonds, in which upon slowing down the cleavage of the other bond a decrease in the magnitudes of the substrate and solvent kinetic isotope effects is expected (*30*). Consistent with a concerted mechanism for the cleavages of the OH and CH bonds, the multiple kinetic isotope effects, with a $^{D,D_2O}k_{red}$ value of ∼9, were not smaller than the product of the individual substrate and solvent kinetic isotope effects, with a value of ∼7 (i.e., $^{D}k_{red} \times {}^{D_2O}k_{red}$) (*30*). In contrast, the Asn510His enzyme catalyzed the cleavage of the substrate OH and CH bonds with a stepwise mechanism, as indicated by the decreases in the substrate and solvent kinetic isotope effects upon slowing down the cleavage of the other bond per effect of deuteration and the $^{D,D_2O}k_{red}$ value of ∼5 being smaller than the product of the individual substrate and solvent kinetic isotope effects, with a value of ∼11 (i.e., $^{D}k_{red} \times {}^{D_2O}k_{red}$) (*30*). Finally, previous studies using kinetic isotope effects established a stepwise mechanism for the cleavages of the OH and CH bonds in the wild-type enzyme (*3*). The change in the timing for the cleavages of the OH and CH bonds of choline catalyzed by the Asn510Ala enzyme probably stems from the mutated enzyme having likely lost the capability of the wild-type and the Asn510His enzymes to form a hydrogen bond interaction between the side chain at position 510 and a reaction intermediate or a transition state or, alternatively, a neighboring residue such as His466 that directly interacts with a reaction intermediate and transition state (*9*).

The effect of the mutation of Asn510 on the reactivity of the reduced flavin with oxygen in choline oxidase is difficult to rationalize with the data at hand. In choline oxidase, activation of oxygen for reaction with the flavin is exerted by the positive charge harbored on the quaternary ammonium group of choline rather than by side chains of histidine and lysine residues as in the case of glucose oxidase and monomeric sarcosine oxidase (*31*−*37*). In this respect, mutation of either His351 or His466 with alanine does not affect the oxidative half-reaction in choline oxidase, as suggested by lack of effects on the k_{cat}/K_{oxygen} values (*9*, *12*). This suggests that the low reactivity of the flavin with oxygen in the mutant enzymes is likely not mediated by the residues neighboring Asn510. An interesting alternative is that the mutation of Asn510 to histidine or alanine may alter the pattern of hydrogen bonds between the protein microenvironment and the flavin, since the side chain of Asn510 is less than 5 Å from the flavin O(2) and N(3) atoms of the isoalloxazine ring. In this respect, replacement of the analogous Asn485 in cholesterol oxidase with leucine has been associated with the loss of a hydrogen bond involving the π-system of the flavin (*22*). Current efforts are aimed at the elucidation of the three-dimensional structures of the Asn510 mutant enzymes by using X-ray crystallography.

Asn510 is important for the flavinylation reaction in which FAD is covalently attached to the protein moiety in choline oxidase. Evidence in support of this conclusion comes from the stoichiometry of FAD to protein in the Asn510 mutant enzymes that is 5-fold lower than that of the wild-type enzyme, where a 1:1 stoichiometry was established (*7*, *24*). Furthermore, upon introducing by mutation a negative charge on residue 510 (i.e., Asn → Asp mutant enzyme), ∼75% of the flavin that is bound to the protein has a noncovalent association. Since Asn510 sits less than 5 Å from the flavin N(1)−C(2) atoms, a ready explanation is that in the Asn510His, Asn510Ala, and Asn510Leu enzymes there is a decreased stabilization of the negative charge that is required on the flavin N(1)−C(2) atoms for the formation of the covalent attachment to the protein (*38*). In agreement with this conclusion is the observation that the anionic flavosemiquinone is not observed during the purification of the Asn510Asp, Asn510Ala, and Asn510Leu enzymes, in contrast to what occurs in the wild-type enzyme (*7*, *24*). In the Asn510Asp enzyme, where the side chain of the residue is negatively charged, such a lack of stabilization of the negative charge on the flavin is even more dramatic, to the extent that the reaction of covalent attachment of the flavin to the protein is also impaired. Lack of stabilization of the negative charge on the reduced flavin is also consistent with the Asn510Asp enzyme being devoid of enzymatic activity with choline, since the enzyme would not be able to stabilize the anionic hydroquinone during turnover with choline. Previously, we have shown that in choline oxidase the replacement of His466 with aspartate also results in an enzyme that is unable to consume oxygen in the presence of choline, has a low stoichiometry of FAD:protein, and has ∼75% of the enzyme-bound flavin being noncovalently linked to the protein (*10*). Collectively, the results strongly support the importance of the protein microenvironment in proximity of the flavin N(1)−C(2) atoms as being important for the flavinylation of the protein in choline oxidase.

In conclusion, the results of the biochemical and mechanistic investigation of active site mutant enzymes where the conserved residue Asn510 is substituted by site-directed mutagenesis with alanine, histidine, leucine, or aspartate are consistent with Asn510 being important for both choline and FAD oxidations. Substitution of Asn510 with alanine or histidine yields enzymes with a significant decrease in the rate of hydroxyl proton abstraction with respect to wild-type choline oxidase. Moreover, the Asn510Ala enzyme lost the ability of the wild-type or the Asn510His enzymes to stabilize the alkoxide intermediate that is formed in the oxidation of choline, displaying a concerted mechanism for the cleavages of the OH and CH bonds of choline. Minimal changes in the K_d values for choline in the Asn510Ala and Asn510His enzymes suggest no direct involvement of Asn510 in substrate binding. Finally, all of the mutations introduced at residue 510 resulted in enzymes with much lower levels of FAD incorporation, with most of the flavin being associated noncovalently upon replacing Asn510 with aspartate. These studies therefore represent an example of how the relative timing for cleavages of multiple bonds in an enzymatic reaction can be modulated depending upon the type of mutation that is introduced at a specific active site position.

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