On the Role of Histidine 351 in the Reaction of Alcohol Oxidation Catalyzed by Choline Oxidase[†]

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ABSTRACT: Choline oxidase catalyzes the four-electron, flavin-linked oxidation of choline to glycine betaine with transient formation of an enzyme-bound aldehyde intermediate. The recent determination of the crystal structure of choline oxidase to a resolution of 1.86 Å established the presence of two histidine residues in the active site, which may participate in catalysis. His466 was the subject of a previous study [Ghanem, M., and Gadda, G. (2005) *Biochemistry 44*, 893−904]. In this study, His351 was replaced with alanine using site-directed mutagenesis, and the resulting mutant enzyme was purified and characterized in its mechanistic properties. The results presented establish that His351 contributes to substrate binding and positioning and stabilizes the transition state for the hydride transfer reaction to the flavin, as suggested by anaerobic substrate reduction stopped-flow data. Furthermore, His351 contributes to the overall polarity of the active site by modulating the pK_a of the group that deprotonates choline to the alkoxide species, as indicated by pH profiles of the steady-state kinetic parameters with the substrate or a competitive inhibitor. Surprisingly, His351 is not involved in the activation of the reduced flavin for reaction with oxygen. The latter observation, along with previous mutagenesis data on His466, allow us to conclude that choline oxidase must necessarily utilize a strategy for oxygen reduction different from that established for glucose oxidase, where other authors showed that the catalytic effect almost entirely arises from a protonated histidine residue.

Choline oxidase (E.C. 1.1.3.17) catalyzes the four-electron, flavin-linked oxidation of choline to glycine betaine with transient formation of an enzyme-bound aldehyde. The overall reaction includes two reductive half-reactions in which a protein-bound flavin accepts a hydride ion from the alcohol substrate (step a in Scheme 1) and the hydrated betaine aldehyde intermediate (step c in Scheme 1). Each reductive half-reaction is followed by an oxidative half-reaction in which a hydride equivalent and a proton are transferred from the reduced flavin and the protein to molecular oxygen with formation and release of hydrogen peroxide (*1–4*). The enzyme-bound aldehyde produced in the first oxidation reaction is rapidly hydrated in the active site of the enzyme to the *gem*-diol species (step b in Scheme 1), which is the form acting as substrate in the second oxidation reaction catalyzed by the enzyme (*2*). The first reductive half-reaction in which choline is oxidized to betaine aldehyde has been studied extensively using a variety of mechanistic, biochemical, mutagenesis, and structural approaches using choline and choline analogues (*1–11*). Catalysis is initiated in the enzyme−substrate complex by a kinetically fast removal of the hydroxyl proton of the alcohol substrate by an as yet unidentified catalytic base with pK_a of ∼7.5 (step a in Scheme 2) as suggested by kinetic isotope effect (*3, 10*). The resulting zwitterionic, choline alkoxide species is stabilized in the enzyme−substrate complex through electrostatic bonds with the charged side chains of His466 and Glu312 at the opposite ends of the molecule (*4, 6, 9*). These interactions, along with the limited mobility of the flavin cofactor that is covalently linked through its CM 8 group to His99 (*9*), likely contribute to preorganize the enzyme−substrate complex for an efficient oxidation reaction (*9*). The hydride transfer reaction between the alkoxide α-carbon and the N(5) atom of the flavin then occurs quantum mechanically as a result of environmental vibrations of the reaction coordinate that permit a tunneling distance between the hydride donor and acceptor within a highly preorganized enzyme-complex Michaelis (steps b and c in Scheme 2) (*8*). The resulting reduced flavin finally reacts with molecular oxygen to yield hydrogen peroxide, before a second oxidation reaction will produce glycine betaine from betaine aldehyde. In agreement with a highly preorganized enzyme−substrate complex, recent mutagenesis studies showed that replacement of Glu312 with aspartate results in a 260-fold decrease in the rate of hydride transfer to the flavin (*9*).

The oxidative half-reaction in which a hydride equivalent and a proton are transferred from the reduced flavin and the protein to molecular oxygen to yield hydrogen peroxide in choline oxidase is less understood. However, this reaction has been well-characterized in another member of the glucose−methanol−choline oxidoreductase enzyme superfamily to which choline oxidase belongs, namely, glucose

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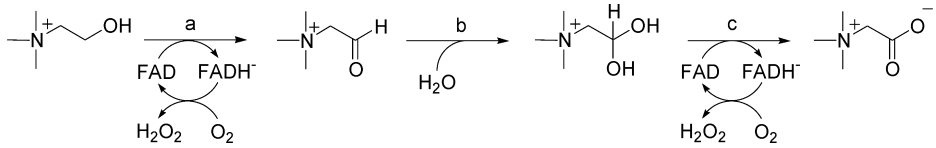
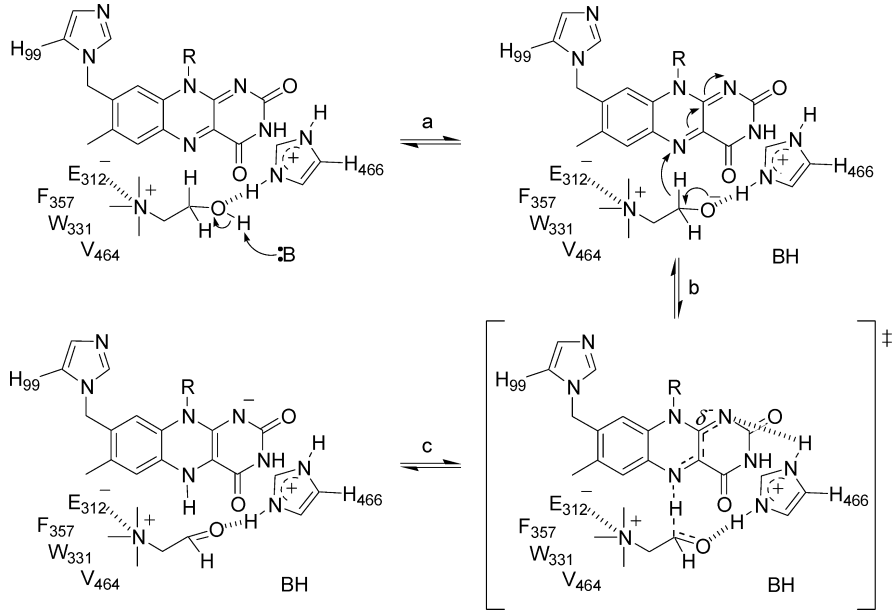
Scheme 1: Reaction Catalyzed by Choline Oxidase



Scheme 2: Oxidation of Choline Catalyzed by Choline Oxidase



oxidase (*12–15*). In that enzyme, several mechanistic probes have been used by other authors on the wild type and a mutant variant of the enzyme in which His516 was replaced with alanine to convincingly conclude that the catalytic effect of glucose oxidase in the reduction of oxygen arises almost entirely from a single positive charge located on His516 (*13*). In this context, previous mechanistic studies indicated that in choline oxidase the reactivity of the reduced flavin toward oxygen is not affected upon replacement of His466 with alanine, which is equivalent to the His516Ala mutation in glucose oxidase (*4*). Moreover, in contrast to the observations reported for glucose oxidase, where there is a marked effect of pH on the reactivity of the reduced flavin with oxygen (*12, 13, 15*), the wild-type form of choline oxidase shows similar reactivity in the pH range from 6 to 10 (*10*). As illustrated in Figure 1, the recent determination of the crystal structure of choline oxidase to a resolution of 1.86 Å shows that a second histidine residue, namely, His351, is located in the active site in a location that suggests its possible involvement in the oxygen reduction reaction. His351 may therefore play catalytic roles similar to those of His516 in glucose oxidase.

The present study was conducted with the dual goal of evaluating whether His351 participates in the reductive and the oxidative half-reactions catalyzed by choline oxidase. In the former His351 could catalyze the abstraction of the hydroxyl proton of the alcohol substrate that results in the activation of choline. In the latter it could provide the necessary stabilization that is required for the reaction of the reduced flavin with oxygen. Toward these goals, His351 was replaced with alanine using site-directed mutagenesis, and the resulting mutant enzyme was purified and characterized in its kinetic and mechanistic properties. The results presented are consistent with His351 being important for the proper binding and positioning of the substrate so that the subsequent hydride transfer reaction occurs efficiently, for stabilization of the transition state developed during choline oxidation, and for fine-tuning the polarity of the active site that is required for choline activation. However, the results of the present study do not support the notions that His351 is solely

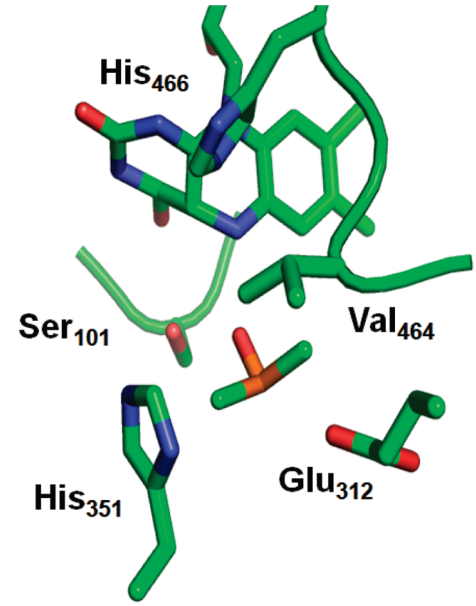


FIGURE 1: The active site of wild-type choline oxidase at a resolution of 1.86 Å (PDB 2jbv). Selected amino acids are shown, along with DMSO, an additive used in the crystallization process. Note the distortion of the flavin ring, which is due to the presence of a C4a flavin adduct (not shown here), whose nature is currently under investigation.

responsible for the abstraction of the hydroxyl proton of choline and that it participates in the activation of the reduced flavin for reaction with oxygen.

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, 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 deuteroxide (99%) were from Isotec Inc. (Miamisburg, OH). Wild-type choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA*1 and purified to homogeneity as described previously (*1*). All other reagents were of the highest purity commercially available.

Site-Directed Mutagenesis. A QuikChange kit was used to prepare the mutant enzyme His351Ala. The experiment was performed by following the manufacturer's instructions in the presence of 2% DMSO using the pET/*codA*mg1 plasmid harboring the wild-type gene encoding for choline oxidase (*codA*) as a template and forward and reverse oligonucleotides as primers for site-directed mutagenesis. The resulting mutant gene (*codA*mg1-H351A) was 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 gene in the correct orientation. Finally, *E. coli* strain Rosetta(DE3)pLysS competent cells were transformed with the mutant plasmid pET/*codA*mg1-H351A by electroporation, and permanent stocks of the transformed cells were prepared and stored at −80 °C.

Expression and Purification of His351Ala Variant of Choline Oxidase. Permanent stocks of *E. coli* Rosetta(DE3)-pLysS cells harboring plasmid pET/*codA*mg1-H351A 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 12−14 h at 37 °C, before inducing protein expression with 0.05 mM IPTG for 5 h at 22 °C. The mutant enzyme was purified to homogeneity using the same procedure described previously for the purification of the wild-type enzyme (*1*).

Enzyme Assays. The enzymatic activity of the His351Ala[1] enzyme variant was measured by the method of initial rates as described for the wild-type enzyme (*1*) using a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.). The steady-state kinetic parameters of the mutant enzyme were determined by varying the concentration of oxygen (in the range from 0.02 to 0.1 mM) and choline (in the range from 0.5 to 50 mM) in 50 mM sodium pyrophosphate, pH 10 and 25 °C. The assay reaction mixture was equilibrated at the desired oxygen concentration by bubbling with a O_2/N_2 gas mixture for at least 15 min before the reaction was started with the addition of the enzyme. Product inhibition studies were carried out by measuring enzymatic activity at varying concentrations of choline at several fixed concentrations of glycine betaine (from 0 to 80 mM) in air-saturated 50 mM sodium pyrophosphate at 25 °C over a pH range of 6−9.5. The pH dependences of the steady-state kinetic parameters of the His351Ala enzyme variant were determined by measuring enzymatic activity at varying concentrations at both organic substrate and oxygen in the pH range between 5 to 10 in 50 mM sodium pyrophosphate at 25 °C, with the exception of pH 7 where sodium phosphate was used. The pH dependence of the primary kinetic isotope effect with 1,2-[$^2H_4$]choline as substrate was determined by measuring the kinetic parameters of the mutant enzyme with labeled and unlabeled substrate in air-saturated 50 mM sodium pyrophosphate at 25 °C in the pH range 7−10. Pre-steady-state kinetic measurements were carried out by using a Hi-Tech SF-61 stopped-flow spectrophotometer thermostated at 25 °C, pH 10. The rate of flavin reduction was measured by monitoring the decrease in absorbance at 453 nm which results from the anaerobic mixing of the mutant enzyme with the organic substrate as described in ref *3* for the wild-type enzyme. The mutant enzyme was mixed anaerobically with an equal volume of substrate, obtaining a final reaction mixture of ∼15 μM enzyme and 0.2−10 mM choline or 1−25 mM betaine aldehyde. For each concentration of substrate, the rates of flavin reduction were recorded in triplicate, with measurements usually differing by ≤5%.

Data Analysis. Kinetic data were fit with the KaleidaGraph (Synergy Software, Reading, PA) and the Enzfitter (Biosoft, Cambridge, U.K.) softwares. Apparent kinetic parameters in atmospheric oxygen were determined by fitting initial rates at different substrate concentration to the Michaelis−Menten equation for one substrate. 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. 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).

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

The pH profiles of the k_{cat} and k_{cat}/K_m values were determined by fitting initial rate data to eq 2, which describes a curve with a slope of +1 and a plateau region at high pH. C is the pH-independent value of the kinetic parameter of interest. Product inhibition data were fit to eq 3, which describes a competitive inhibition mechanism of the organic substrate and the product. K_{is} is the inhibition constant and P is the concentration of glycine betaine. The pH profile of enzyme inhibition by glycine betaine was determined by fitting the initial rate data to eq 4, which describes a curve with a slope of −1 and a plateau region at low pH. Kinetic isotope effects were determined by taking the ratio of the kinetic parameters obtained with choline to that obtained with 1,2-[$^2H_4$]choline.

[1] Abbreviations: His351Ala enzyme, choline oxidase variant with His351 replaced with alanine; His466Ala enzyme, choline oxidase variant with His466 replaced with alanine.

$$\log Y = \log \frac{C}{1 + \frac{10^{-\mathrm{pH}}}{10^{-\mathrm{p}K_a}}} \quad (2)$$

$$\frac{v}{e} = \frac{k_{cat}A}{K_a\left(1 + \frac{P}{K_{is}}\right) + A} \quad (3)$$

$$\log Y = \log \frac{C}{1 + \frac{10^{-\mathrm{p}K_a}}{10^{-\mathrm{pH}}}} \quad (4)$$

Stopped-flow data traces were fit with eq 5, which describes a single-exponential process, in which k_{obs} is the apparent 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. Pre-steady-state kinetic parameters were determined by using eq 6, where k_{obs} is the observed first-order rate 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.

$$A = B\exp(-k_{obs}t) + C \quad (5)$$

$$k_{obs} = \frac{k_{red}A}{K_d + A} \quad (6)$$

RESULTS

Purification of the His351Ala Variant of Choline Oxidase. The His351Ala variant of choline oxidase in which His351 was replaced with alanine was engineered using site-directed mutagenesis, expressed in *E. coli* strain Rosetta(DE3)pLysS and purified by anionic exchange chromatography at pH 8 using the same protocol established for the wild-type enzyme (*1*). The purified enzyme had about 50−70% of the enzyme-bound flavin cofactor as an air-stable anionic flavosemiquinone, which was slowly converted to the oxidized state by extensive dialysis at pH 6 and 4 °C (Figure S1 in Supporting Information). In this respect, the His351Ala enzyme behaved like the wild type, which was previously shown to stabilize the anionic flavosemiquinone in air at pH 8 (*1, 10*). The UV−visible absorbance spectrum of the oxidized form of the His351Ala variant at pH 8 showed peaks at 367 and 454 nm with an ϵ_{454nm} value of 11.7 mM^{-1} cm^{-1}, in agreement with the value of 11.4 mM^{-1} cm^{-1} previously observed with the wild-type enzyme (*10*). Acid denaturation of the enzyme using 10% TCA, followed by centrifugation to remove the unfolded protein, yielded a supernatant that was devoid of absorbance in the visible region of the spectrum, consistent with the flavin cofactor being covalently bound to the protein. The fully oxidized form of the His351Ala enzyme had a specific activity of 0.04 μmol O_2 min^{-1} mg^{-1} with 10 mM choline as substrate at pH 7, which was ~200-fold lower than that of the wild-type enzyme, suggesting that His351 is important for catalysis in choline oxidase.

Steady-State Kinetics. The steady-state kinetic parameters of the His351Ala enzyme were determined using choline as substrate at 25 °C by monitoring the rate of oxygen consumption at varying concentrations of both the organic substrate and oxygen. The determination was carried out at pH 10 because all of the kinetic parameters for the wild-type (*10*) and the His351Ala mutant enzymes (this study) were independent of the pH in this region, thereby allowing for a direct comparison of the values obtained with the two enzymes. As for the case previously reported for the wild-type form of choline oxidase (*3*), the best fits of the data were obtained with eq 1, consistent with the order of the kinetic steps involving substrate binding and product release being the same in the two enzymes. Substitution of histidine with alanine at position 351 resulted in a decrease in the overall turnover with choline of 60-fold with respect to the turnover of the wild-type enzyme (Table 1). A 360-fold decrease in the k_{cat}/K_m value with choline was also observed, suggesting that the histidine to alanine mutation affects the reductive half-reaction in which the organic substrate is oxidized by choline oxidase. In contrast, the k_{cat}/K_{O_2} value, which directly reports on the oxidative half-reaction in which the reduced flavin reacts with molecular oxygen (*16*), was only 1.2 times lower than in the wild-type enzyme (*10*). These data are consistent with His351 playing a minimal role in the oxidative half-reaction in which the reduced flavin reacts with molecular oxygen.

Table 1: Comparison of the Kinetic Parameters of His351Ala[a] and Wild-Type[b] Choline Oxidase at pH 10

	kinetic parameter	His351Ala	wild type
choline	k_{cat}, s^{-1}	1.00 ± 0.01	60 ± 1
	K_m, mM	1.50 ± 0.30	0.25 ± 0.01
	K_{O_2}, μM	16 ± 2	690 ± 30
	k_{cat}/K_m, M^{-1} s^{-1}	660 ± 120	237000 ± 9000
	k_{cat}/K_{O_2}, M^{-1} s^{-1}	70000 ± 10000	86400 ± 3600
	K_{ia}, mM	7.1 ± 1.6	0.14 ± 0.01
	k_{red}, s^{-1}	1.2 ± 0.1	93 ± 1
	K_d, mM	2.6 ± 0.3	0.29 ± 0.01
	$^D(k_{cat}/K_m)$	7.8 ± 0.2	10.7 ± 2.6
	$^Dk_{cat}$	5.0 ± 0.2	7.5 ± 0.3
betaine aldehyde	k_{red}, s^{-1}	4.4 ± 0.4	135 ± 4
	K_d, mM	12.0 ± 2.2	0.45 ± 0.03

[a] Enzymatic activity was measured at varying concentrations of both organic substrate and oxygen in 50 mM sodium pyrophosphate, pH 10, at 25 °C. [b] From ref *3*. The steady-state and pre-steady-state kinetic parameters were determined by using eqs 1 and 6, respectively.

Reductive Half-Reactions with Choline or Betaine Aldehyde. The reductive half-reactions with choline or betaine aldehyde as substrate were investigated further in a stopped-flow spectrophotometer by mixing anaerobically the His351Ala enzyme with different concentrations of choline or betaine aldehyde at pH 10 and 25 °C. In all cases, the decrease in absorbance at 453 nm associated with the reduction of the enzyme-bound flavin was fit best to a single exponential process, as illustrated in the example of Figure 2A. Full reduction of the enzyme-bound flavin to the neutral hydroquinone species was observed in all cases, as suggested by the poorly defined absorbance peak in the 350 nm region of the UV−visible absorbance spectrum and the prominent shoulder centered at 400 nm (Figure 2B). The observed rates of flavin reduction (k_{obs}) were hyperbolically dependent on the concentration of the organic substrate (Figure 2C,D), which allowed for the determination of the limiting rate constants for flavin reduction (k_{red}) and the macroscopic equilibrium constants for substrate binding at the active site

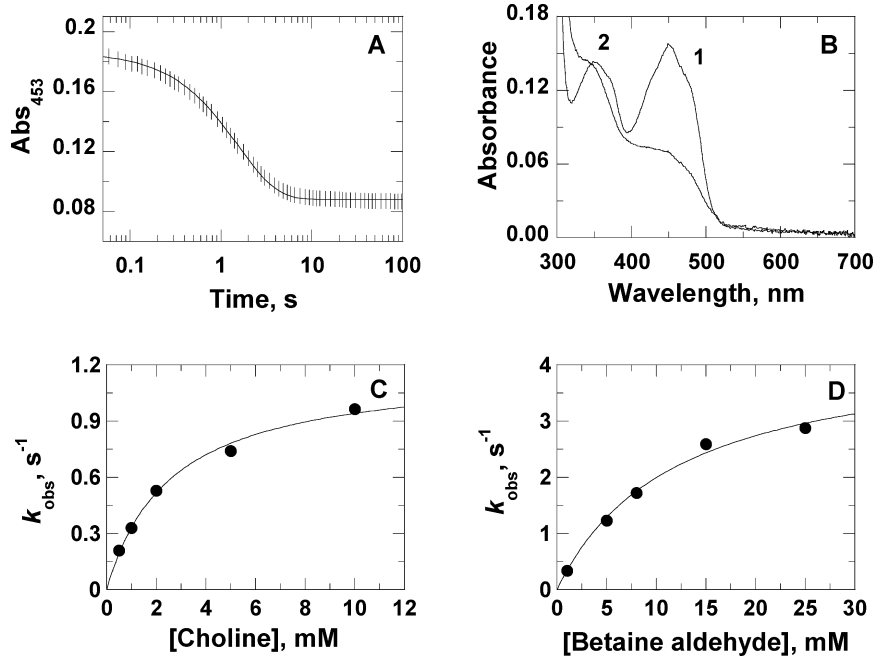


FIGURE 2: Anaerobic substrate reduction of the His351Ala enzyme with choline and betaine aldehyde as substrate. Panel A: Stopped-flow trace obtained upon mixing anaerobically the His351Ala enzyme variant with 15 mM betaine aldehyde in 50 mM sodium phosphate, sodium pyrophosphate buffer, pH 10, at 25 °C. The curve represents the fit of the data with eq 5. Time indicated is after the end of flow, i.e., 2.2 ms. For clarity, one experimental point every 10 is shown (vertical lines). Panel B: UV−visible absorbance spectra of the oxidized (curve 1) and reduced (curve 2) species of the His351Ala enzyme variant obtained by mixing anaerobically the oxidized enzyme with buffer and 10 mM choline in 50 mM sodium phosphate, sodium pyrophosphate buffer, pH 10, at 25 °C. Panels C and D: Observed rates of anaerobic flavin reduction (k_{obs}) as a function of the concentrations of choline and betaine aldehyde, respectively. Data were fit to eq 6.

of the enzyme (K_d). As summarized in Table 1, the k_{red} values for the His351Ala enzyme were 80- and 30-fold lower with choline and betaine aldehyde with respect to the wild-type enzyme. In addition, replacement of His351 with alanine resulted in 10- and 25-fold increases in the K_d values for choline and betaine aldehyde. Thus, His351 plays significant roles for both substrate binding and the hydride transfer reaction catalyzed by choline oxidase.

pH Dependence of the k_{cat} and k_{cat}/K_m Values. The pH profiles of the steady-state kinetic parameters of the His351Ala enzyme were determined in the pH range from 5 to 10, at varying concentrations of both choline and oxygen. Both the k_{cat} and k_{cat}/K_m values with choline increased with increasing pH and reached limiting values at high pH (Figure 3A,B), consistent with the requirement for an unprotonated group in the reductive half-reaction of the mutant enzyme. The apparent pK_a values determined from the plots of log k_{cat}/K_m and k_{cat} versus pH were 7.8 ± 0.2 and 6.6 ± 0.1. For comparison, the wild-type enzyme was previously shown to have pK_a values of 7.6 and 7.1 in the k_{cat}/K_m and k_{cat} pH profiles with choline as substrate (*10*).

In the pH range from 7 to 10 the K_m values for oxygen were all less than 15 μM (Table S2 in Supporting Information), yielding pH-independent k_{cat}/K_{O_2} values in the lower 10^5 M^{-1} s^{-1} range. At pH lower than 7 the K_m values for oxygen decreased further, thereby preventing the determination of the k_{cat}/K_{O_2} values at low pH due to the enzyme being fully saturated with oxygen at the lowest concentrations of oxygen attainable in the steady-state experiments, i.e., 10 μM.

Substrate Kinetic Isotope Effects. Due to the His351Ala enzyme being saturated with oxygen under atmospheric condition, the kinetic isotope effects on the steady-state kinetic parameters were determined with 1,2-[$^2H_4$]choline in air-saturated buffer. As shown in Figure 4, both the $^D(k_{cat}/K_m)$ and $^Dk_{cat}$ values with the His351Ala enzyme were pH-independent in the pH range from 7 to 10, with average values of 7.8 ± 0.2 and 5.0 ± 0.2, respectively. These values were only slightly lower than those of 10.6 and 7.5 previously reported for the wild-type enzyme (*3*), for which the cleavage of the CH bond of choline was shown to be fully rate-limiting for the reductive half-reaction and partially rate-limiting for the overall turnover of the enzyme along with the subsequent step of CH bond cleavage of the aldehyde intermediate (*3*).

Binding of Glycine Betaine. Previous studies showed that glycine betaine inhibits the wild-type form of choline oxidase by binding at the active site of enzyme, with a limiting K_{is} value of 13 mM at low pH and a pK_a value of 7.5 (*10, 11*). Consequently, the pH dependence of the inhibition by glycine betaine was determined to obtain the thermodynamic pK_a value for the group in the active site of the His351Ala enzyme that participates in the reductive half-reaction. As shown in Figure 3C, His351Ala enzyme inhibition was maximal at low pH (K_{is} value of 21 ± 3 mM) and decreased with increasing pH, yielding a pK_a value of 8.0 ± 0.1. Thus, replacement of His351 with alanine resulted in a 0.5 unit increase in the macroscopic, thermodynamic pK_a value for the ionizable group(s) in the active of the enzyme.

DISCUSSION

The variant of choline oxidase in which His351 has been replaced with alanine was prepared in this study to investigate the role played by this residue in the enzyme-catalyzed reaction of choline oxidation. The mutant enzyme is properly folded and is similar to the wild-type enzyme with respect

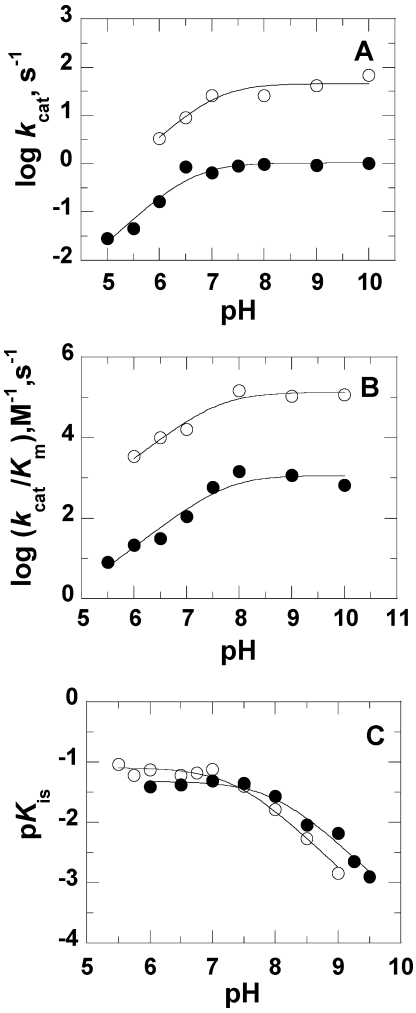


FIGURE 3: pH dependence of the k_{cat} (panel A) and k_{cat}/K_m values for choline (panel B) and glycine betaine inhibition (panel C) for the His351Ala (●) and the wild-type (○) enzymes. Panels A and B: Activity assays were measured at varying concentrations of both choline and oxygen between pH 5 and 10, at 25 °C. The data were fit with eq 2. Panel C: Activity assays were measured at varying concentrations of choline and glycine betaine in air-saturated buffer between pH 6 and 9.5, at 25 °C. The lines were fit of the data to eq 4. The K_{is} value for glycine betaine was determined by fitting the initial rate data to eq 3. Data for the wild-type enzyme are from ref *10*.

to (a) a flavin covalently linked to the protein, (b) the stabilization of an air-stable, anionic flavosemiquinone, which can be slowly oxidized upon prolonged treatment at pH 6 and 4 °C, (c) a sequential steady-state kinetic mechanism in which oxygen reacts in a second-order fashion with the enzyme-bound reduced flavin before the release of the organic product of reaction, (d) a chemical step for cleavage of the substrate CH bond that is fully rate limiting for the reductive half-reaction and partially rate limiting for the overall turnover of the enzyme, (e) the requirement for an unprotonated group in the reductive half-reaction, and (f) the absence of ionizable groups with pK_a in the range from 7 to 10 for the oxidative half-reaction with choline as substrate. Consequently, the comparison of the mechanistic properties that differ from and are similar to one another in the His351Ala and wild-type enzymes can be used to gain insights on the catalytic role exerted by His351 in the active site of choline oxidase.

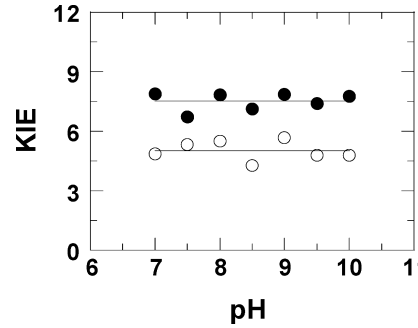


FIGURE 4: pH dependence of substrate deuterium isotope effects on the k_{cat}/K_m values (●) and k_{cat} (○) values with choline as substrate for the His351Ala enzyme variant. Enzymatic activities were measured in air-saturated 50 mM sodium pyrophosphate buffer with choline and 1,2-[$^2H_4$]choline as substrates between pH 7 and 10, at 25 °C. Data were fit by $y = 7.78$ and $y = 5.15$ for k_{cat}/K_m and k_{cat} values, respectively.

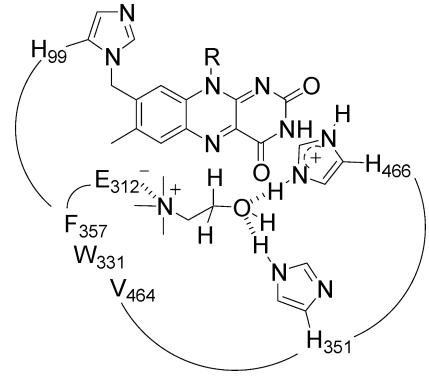


FIGURE 5: Proposed hydrogen-bonding interaction of His351 with choline in the active site of choline oxidase.

The active site residue His351 is important for substrate binding, most likely by acting as a hydrogen bond donor to the hydroxyl oxygen of the alcohol substrate, as illustrated schematically in Figure 5. Evidence supporting this conclusion comes from anaerobic substrate reduction experiments using a stopped-flow spectrophotometer, showing that in the His351Ala variant the K_d values for choline and betaine aldehyde binding are 9 and 17 times larger than the corresponding values in the wild-type enzyme (*10*). The decreases in the chemical affinity of the His351Ala enzyme variant for the substrates correspond to an energetic contribution of the side chain of His351 of 5−8 kJ mol^{-1}, which agrees well with the expected value for a hydrogen-bonding interaction (*17*). Structural data independently support an involvement of His351 in binding to the hydroxyl moiety of the alcohol substrate(s). Indeed, the crystal structure of choline oxidase resolved to 1.86 Å shows that His351 is 3.5 Å from the methyl groups of DMSO, an additive that was used in the crystallization solution and that was proposed to mimic the substrate, and 6.7 Å from the N(5) atom of the flavin (*9*), which is the site acting as the acceptor of the hydride ion originating from the α-carbon of the choline alkoxide species in the reaction catalyzed by the enzyme (Scheme 2) (*3*).

His351 facilitates the hydride transfer reaction between the activated alkoxide intermediate and the flavin N(5) atom (Scheme 2), likely by hydrogen bonding to the oxygen atom of the alkoxide species in the transition state for the reaction. This conclusion is supported by the 75-fold decrease in the rate constant for flavin reduction (k_{red}) that accompanies the replacement of His351 with alanine determined in anaerobic substrate reduction experiments using a stopped-flow spec-

trophotometer. A decrease of 30-fold in the k_{red} value is also observed when hydrated betaine aldehyde is used instead of choline as substrate, further consistent with His351 stabilizing the transition state for the reaction. In agreement with this conclusion the substrate kinetic isotope effect associated with CH bond cleavage is ~30% lower than in the wild-type enzyme, with a $^{D}(k_{cat}/K_m)$ value of 7.8 as compared to 10.6 (*3*). Recent mechanistic studies on the wild-type form of choline oxidase showed that the hydride transfer reaction occurs quantum mechanically within a highly preorganized active site, with little independent movement of the alkoxide species acting as hydride donor and the N(5) atom of the flavin acting as hydride acceptor (*8*). In this context, it is attractive to hypothesize that the decreased rates of hydride transfer with choline and hydrated betaine aldehyde seen in the His351Ala enzyme variant may originate from disruption of preorganization in the enzyme−substrate complex, which would allow for the alkoxide donor and the flavin acceptor more independent movement in the His351Ala enzyme−substrate complex with respect to the wild-type enzyme. This hypothesis is the focus of a current investigation of the enzyme.

Replacement of the histidine residue at position 351 with alanine results in the increase from 7.5 to ~8 of the thermodynamic pK_a value for the catalytic base that activates choline to the alkoxide species, as indicated by the pH profile of glycine betaine inhibition with the His351Ala enzyme. A larger increase in the thermodynamic pK_a value for the catalytic base of choline oxidase, to a value of ~9, was previously observed upon replacing the only other histidine residue located in the active site of the enzyme with alanine (e.g., His466) (*4*). These data are consistent with both active site histidine residues contributing to the overall polarity of the active site, which is essential for an efficient deprotonation of the hydroxyl proton of the alcohol substrate during enzymatic turnover. However, neither of the two histidine residues is essential for the deprotonation reaction (Scheme 2), since the pH profiles for the k_{cat}/K_m values unequivocally show the requirement for a catalytic base in both the His351Ala (this study) and the His466Ala enzyme variants (*4*). Since no ionizable side chains other than His351 and His466 are present in the active site of the enzyme, a rationale that explains the site-directed mutagenesis results is that either one of the two histidines acts as catalytic base in the wild-type enzyme, whereas the other residue would pick up the catalytic role in the absence of the "original" base in the site-directed mutagenized enzyme. Alternatively, one should contemplate the possibility of multiple residues in the active site of the enzyme acting synergistically, and not singlehandedly, to promote acidification of the substrate hydroxyl group through electrostatic and hydrogen bonds. In the active site of choline oxidase, such a synergistic role may be played by Ser101, His351, His466, possibly the C4 oxygen atom of the flavin, and water. In the latter case, the quest for the "catalytic base" using a classical site-directed mutagenesis approach in which a single residue is selectively replaced and the function of the resulting enzyme is investigated would be hampered by the fact that the typical behavior associated with the presence of a catalytic base would still be observed in the pH profiles irrespective of the site of mutagenesis, as has been the case for the His351 and His466 mutant forms of choline oxidase.

His351 does not participate in the activation of the reduced flavin for reaction with molecular oxygen, as demonstrated by the minimal decrease of 1.2-fold in the k_{cat}/K_m value for oxygen in the His351Ala enzyme as compared to the wild-type enzyme (*10*). Interestingly, a similar decrease of 1.5-fold was recently reported in the k_{cat}/K_m value for oxygen for another active site variant of choline oxidase in which the conserved His466 was replaced with alanine (*4*). These results, along with the lack of pH dependence of the k_{cat}/K_m value for oxygen reported in this study for the His351Ala enzyme and in a previous study for the wild-type form of choline oxidase (*10*), are in stark contrast with a mechanistic model recently presented by Klinman and co-workers for the reaction of reduced flavoproteins with oxygen (*13, 15*). Based on studies using a variety of mechanistic probes on the wild-type and an active site mutant form of glucose oxidase in which His516 was replaced by alanine, which is equivalent to the His466 to alanine substitution in choline oxidase, the authors concluded that "virtually all of the catalytic effect in the glucose oxidase reduction of oxygen can be assigned to a single protonated histidine" (*18*). Although the features that activate the reduced flavin for reaction with oxygen in choline oxidase have not been yet elucidated, at this stage of the investigation it can be confidently concluded that a molecular mechanism different from that proposed for glucose oxidase must necessarily operate in choline oxidase. Caution should therefore be exerted in extending *a priori* conclusions that apply to well-characterized systems, such as glucose oxidase, to other oxidases that reduce oxygen in the absence of metal ions.

The neutral hydroquinone species of the flavin is stabilized in the His351Ala enzyme at pH 10, as suggested by the UV−visible absorbance spectrum of the enzyme observed after anaerobic reduction with choline in the stopped-flow spectrophotometer. Stabilization of the neutral flavin hydroquinone at high pH was previously observed in other variants of choline oxidase in which His466 was replaced with alanine or aspartate (*6*). These results contrast to the behavior of the wild-type form of choline oxidase, which stabilizes the anionic hydroquinone species between pH 6 and 10 (*6*). All taken together, these data are consistent with minimal difference in the reactivity of the neutral and anionic flavin hydroquinone species with molecular oxygen, as suggested by the similar k_{cat}/K_m values for oxygen observed in the wild-type (*10*), His351Ala (this study), and His466Ala (*4*) enzymes. Instead, a major determinant for activation of the reduced flavin for reaction with molecular oxygen in choline oxidase is the positively charged trimethylammonium moiety of the enzyme-bound organic substrate, as previously reported (*7, 11*).

The large, pH-independent substrate kinetic isotope effect of 7.8 determined in this study on the second-order rate constant k_{cat}/K_m with 1,2-[$^2H_4$]choline as substrate is consistent with the chemical step of hydride ion transfer from the alkoxide substrate to the flavin being the slowest step in the reductive half-reaction catalyzed by the His351Ala enzyme (*3*). Independent evidence for choline being a slow substrate for the His351Ala enzyme comes from the similarity between the thermodynamic pK_a value for the catalytic group in the active site of the enzyme determined in the pH profile of glycine betaine inhibition, with a value of 8.0 ± 0.1, and the kinetic pK_a value of 7.8 ± 0.2 determined from the pH profile of the k_{cat}/K_m value with choline. In this

respect, the His351Ala enzyme behaves like the wild-type enzyme, for which it was previously shown that the chemical step of CH bond cleavage is fully rate limiting for the reductive half-reaction (*3*).

The overall turnover in the His351Ala enzyme with choline as substrate is limited primarily by the chemical step of hydride ion transfer from the alcohol substrate to the enzyme-bound flavin, with a minor contribution of the second hydride ion transfer reaction involving the aldehyde intermediate and the flavin. This conclusion is supported by both steady-state kinetics and anaerobic substrate reduction data, showing that the k_{cat} value of 1 s^{-1} experimentally determined at pH 10 is approximated fairly well by eq 7, where $k_{red(CH)}$ represents the limiting rate constant for hydride transfer reaction between choline and the enzyme-bound flavin, with a value of 1.2 ± 0.1 s^{-1}, and $k_{red(BA)}$ is the corresponding limiting rate constant for the hydride transfer reaction from the aldehyde intermediate to the flavin, with a value of 4.4 ± 0.4 s^{-1}.

$$k_{cat} = \frac{k_{red(CH)}k_{red(BA)}}{k_{red(CH)} + k_{red(BA)}} = 0.94 \pm 0.11\ s^{-1} \qquad (7)$$

In summary, the results presented in this study on the mutant form of choline oxidase in which His351 has been replaced with alanine allow to support the notion that His351 is an important, but not an essential, residue for the reductive half-reaction of choline oxidation catalyzed by choline oxidase. In contrast, His351 has a minimal role in the subsequent oxidative half-reaction in which the reduced flavin reacts with oxygen. In the reductive half-reaction, His351 contributes to substrate binding by providing a hydrogen bond with its imidazole side chain to the hydroxyl group of choline. This hydrogen-bonding interaction is important for the subsequent reaction of hydride transfer from the α-carbon of the activated, alkoxide substrate to the N(5) atom of the enzyme-bound flavin, in that it most likely contributes to the correct positioning of the hydride donor and acceptor in the enzyme−substrate complex. In this respect, the large substrate kinetic isotope effect seen here on the k_{cat}/K_m value is an important prerequisite for future mechanistic investigations aimed at the elucidation of the importance of His351 in the hydride transfer reaction catalyzed by choline oxidase. Furthermore, His351 contributes to the overall polarity of the active site, by modulating the macroscopic pK_a value for the group that activates choline to the alkoxide species, whose identity still remains elusive. Finally, neither His351 nor His466, which are the only ionizable residues in the active site of the enzyme with the potential to gain a positive charge, plays a role in the reaction between the reduced flavin and molecular oxygen. Thus, important questions that pertain to the molecular mechanisms for alkoxide formation by the enzyme and oxygen reduction by the flavin will have to be addressed in future investigations.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 shows the UV−visible absorbance spectrum of the His351Ala enzyme as purified. Table S1 shows the observed rates and amplitudes of the changes at 453 nm associated with the anaerobic flavin reduction of the His351Ala enzyme with choline or betaine aldehyde as substrate at pH 10, 25 °C. Table S2 shows the steady-state kinetic parameters with choline as a substrate for the data presented in Figure 3A,B. Table S3 shows the apparent steady-state kinetic parameters for the data presented in Figure 3C. This material is available free of charge via the Internet at http://pubs.acs.org.

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