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# Identification of the Catalytic Base for Alcohol Activation in Choline Oxidase

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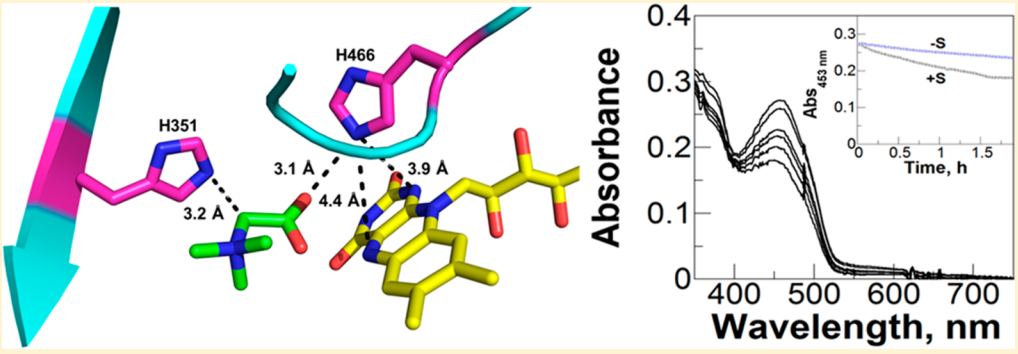


**ABSTRACT:** Choline oxidase catalyzes the oxidation of choline to glycine betaine through a two-step, four-electron reaction with betaine aldehyde as an intermediate. Oxygen is the final electron acceptor. Alcohol oxidation is initiated by the removal of the substrate hydroxyl proton by an unknown active site residue with a p$K_a$ value of ∼7.5. In the crystal structure of the enzyme in complex with glycine betaine, H466 is ≤3.1 Å from the carboxylate oxygen of the reaction product, suggesting a possible role in the proton abstraction reaction catalyzed by the enzyme. H466, along with another potential candidate, H351, was previously mutated to alanine, but this failed to establish if either residue was involved in activation of the substrate. In this study, single variants of choline oxidase with H466 and H351 substituted with glutamine were prepared, purified, and characterized. The $k_{cat}$ and $k_{cat}/K_m$ values of the H351Q enzyme in atmospheric oxygen were 45- and 5000-fold lower than those of the wild-type enzyme, respectively, whereas the H466Q enzyme was inactive when assayed polarographically with choline. In the H466Q enzyme, the rate constant for anaerobic flavin reduction ($k_{red}$) with choline was 1 million-fold lower than in the wild-type enzyme. A comparison of the fluorescence, circular dichroism, and $^1$H nuclear magnetic resonance spectroscopic properties of the H466Q enzyme and the wild-type enzyme is consistent with the mutation not affecting the topology of the active site or the overall fold of the protein. Thus, the change in the $k_{red}$ value and the lack of oxygen consumption upon mutation of histidine to glutamine are not due to misfolded protein but rather to the variant enzyme being unable to catalyze substrate oxidation. On the basis of the kinetic and spectroscopic results presented here and the recent structural information, we propose that H466 is the residue that activates choline to the alkoxide for the subsequent hydride transfer reaction to the enzyme-bound flavin.

Choline oxidase (EC 1.1.3.17) is a dimeric, FAD-dependent enzyme that catalyzes the oxidation of choline to glycine betaine through two consecutive, flavin-mediated redox reactions (Scheme 1). Molecular oxygen oxidizes the reduced enzyme, forming hydrogen peroxide.[1−3] The enzyme from *Arthrobacter globiformis* has been characterized through site-directed mutagenesis, steady-state and rapid reaction kinetics, X-ray crystallography, and computational methods,[1,3−7] allowing for a description of the mechanism of alcohol oxidation at the molecular level. Briefly, choline enters the active site using a gated mechanism controlled by a cluster of hydrophobic residues (M62, L65, V355, F357, and M359) located on the solvent accessible surface of the enzyme.[4] The side chain of E312 establishes an electrostatic interaction with the trimethylammonium group of the substrate, contributing to optimal positioning of the substrate for catalysis.[8] Oxidation of the substrate in the enzyme−substrate complex proceeds through an initial deprotonation of the hydroxyl group that yields an alkoxide from which a rate-limiting hydride transfer to the flavin N(5) atom occurs.[1] The hydride ion tunnels from the alkoxide C$^\alpha$ atom to the flavin N(5) atom, within a highly preorganized enzyme−alkoxide complex.[9] Electrostatic interactions between the protein side chains of residues E312 and H466 and the substrate trimethylamine and O atom of the alkoxide, along with the covalent linkage of the flavin to H99, contribute to the preorganization of the substrate and the flavin.[5,8−11] Several other protein side chains contribute to catalysis or substrate positioning, including S101,[12] H351,[13] and N510.[14] Although the involvement of a

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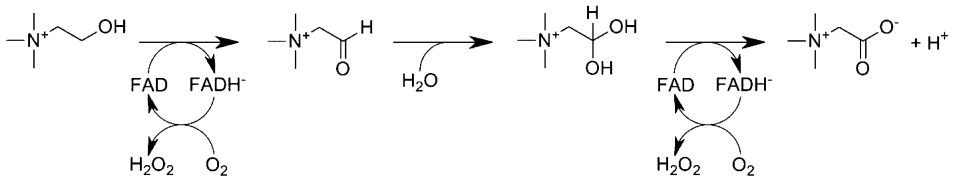
**Scheme 1. Oxidation of Choline Catalyzed by Choline Oxidase**




catalytic base with a p$K_a$ of 7.5 has been demonstrated using steady-state and rapid reaction kinetics in wild-type[1,2] and several variant enzymes,[8,13,15] its identity has not yet been established.

Previous studies that aimed to identify the base in choline oxidase initially focused on H466, primarily because it is fully conserved in the glucose−methanol−choline oxidoreductase enzyme superfamily,[16−20] is 4.4 Å from the flavin N(5) atom, and is equivalent to H502 of aryl-alcohol oxidase[16,21] and H548 of pyranose 2-oxidase,[17,19] which were recently shown to act as catalytic bases through mechanistic, structural, and computational studies. When H466 of choline oxidase was mutated to alanine, there was a 60-fold decrease in the $k_{cat}$ value, while the pH profile of the $k_{cat}/K_m$ value indicated that a base was still present in the variant enzyme.[15] Similar results were obtained upon replacing H351 with alanine in the active site of the enzyme.[13] The decreased catalytic activity observed in the H466A variant was due to a decrease in the electrophilicity of the enzyme-bound FAD and the inability of the enzyme to stabilize the negatively charged alkoxide species formed in catalysis after substrate activation.[15] The latter conclusion was based on partial rescuing of the $k_{cat}$ value with choline in the H466A enzyme by exogenous imidazolium, rather than imidazole, consistent with the side chain of H466 being protonated in the rate-limiting step for overall enzyme turnover, i.e., during the transfer of the hydride from the alkoxide to the flavin.[15] On the basis of these results, the conclusion that neither H351 nor H466 is likely to be the base that deprotonates choline for subsequent hydride transfer in the active site of the enzyme was reached.[13,15,22] The positive charge on H466 was reversed by engineering an H466D variant of the enzyme, which produced an inactive enzyme unable to stabilize the negative charge on the reduced flavin.[22] The presence of the negatively charged aspartate altered the midpoint redox potential of the flavin from 73 mV in the wild type to −89 mV in the variant and resulted in partial incorporation of the flavin (i.e., ∼0.3 mol of FAD/mol of enzyme) with the majority (i.e., ∼75%) being noncovalently bound.[22]

Our recent determination of the crystal structure of choline oxidase in a complex with glycine betaine to a resolution of 1.95 Å shows that the $N^{\varepsilon 2}$ atom of the H466 side chain is ≤3.1 Å from one of the O atoms of the ligand carboxylate (Figure 1).[6] In contrast, the side chain of H351 is ≤3.2 Å from the $C^{\alpha}$ atom of glycine betaine, which corresponds to the $C^{\beta}$ atom of choline before its oxidation by the enzyme (Figure 1). The structural data are consistent with mechanistic data showing that H351 is important for substrate binding and positioning, and it contributes to the stabilization of the transition state for the hydride transfer reaction.[6,13] However, on the basis of the structural information, Salvi et al. suggest that H466 may be the base required for alkoxide formation in the catalytic mechanism of choline oxidase. Reexamination of the mechanistic data for the H466A variant would support such a hypothesis. A possible explanation that reconciles the structural and mechanistic data is that hydroxide ion occupying the space vacated by the missing

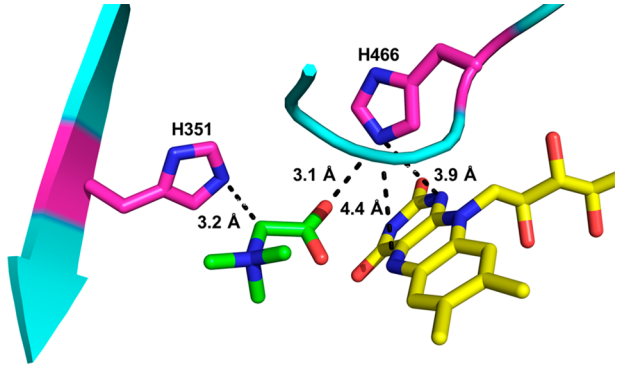



**Figure 1.** Interactions of the histidine residues at positions 466 and 351 of wild-type choline oxidase in complex with the reaction product, glycine betaine (green) (Protein Data Bank entry 4MJW).[6]

side chain in the H466A enzyme may act as a poor surrogate base in catalysis. On the basis of structural considerations alone, space for hydroxide ion is available in the alanine variant that replaces the histidine at position 466. The positive charge on the trimethylammonium headgroup of the substrate bound in the active site of the enzyme would be a primary effecter for lowering the basicity of water, thereby increasing the concentration of hydroxide ion in the active site of the mutant enzyme, as previously established in model systems.[23−25]

In this study, we have replaced H351 and H466 with glutamine to further address the question of which residue acts as a base in the oxidation reaction catalyzed by choline oxidase. We reasoned that in the tight and highly preorganized active site of the enzyme in complex with the substrate a histidine to glutamine replacement would not leave sufficient space for water to intervene in catalysis. While the H351Q enzyme was able to oxidize choline in a standard polarographic assay, although with $k_{cat}$ and $k_{cat}/K_m$ values significantly lower than those of the wild-type enzyme, the H466Q enzyme was completely devoid of enzymatic activity. The results of the mechanistic and spectroscopic studies presented herein allowed us to identify H466 as the residue that acts as the catalytic base in the active site of choline oxidase, thereby providing new insights into the chemical mechanism of the enzyme.

## EXPERIMENTAL PROCEDURES

**Materials.** *Escherichia coli* strain Rosetta(DE3)pLysS was from Novagen (Madison, WI). DNase was purchased from Roche (Indianapolis, IN). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The QIAprep Spin Miniprep kit was from Qiagen (Valencia, CA). Oligonucleotides were synthesized by Sigma Genosys (The Woodlands, TX). Choline, glucose, bovine serum albumin, chloramphenicol, ampicillin, dimethyl sulfoxide, isopropyl β-D-thiogalactopyranoside, phenylmethanesulfonyl fluoride, Luria-Bertani agar and broth, and lysozyme were from Sigma-Aldrich (St. Louis, MO). EDTA and glycerol were from Fisher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(Pittsburgh, PA). Deuterium oxide was obtained from Cambridge Isotope Co. (Andover, MA). All other reagents were of the highest purity commercially available.

**Site-Directed Mutagenesis.** A QuikChange kit was used to prepare the H351Q and H466Q enzyme variants. Mutagenesis was performed by following the manufacturer's instructions in the presence of 2% DMSO to overcome the high GC content in the gene by using the pET20b(+) plasmid harboring the wild-type gene (Cod*A*) as a template. The presence of the mutation was confirmed by sequencing at the DNA core facility of Georgia State University using an Applied Biosystems Big Dye Kit on an Applied Biosystems model ABI 377 DNA sequencer. Finally, *E. coli* strain Rosetta(DE3)pLysS competent cells were transformed with the plasmids containing the mutated genes, e.g., pET20b-(+)/*codA*-H466Q, and permanent stocks of the cells were prepared and stored at −80 °C.

**Enzyme Purification.** The H466Q and H351Q enzymes were expressed and purified to high levels using methods previously described for the wild-type enzyme with the addition of 10% (v/v) glycerol to all the buffers used for purification to increase enzyme stability and minimize potential loss of flavin.[26−28] The H99N variant enzyme was expressed and purified to high levels as previously described by Quaye et al.[5] Fully oxidized variant enzymes were prepared as described by Gadda et al.[26]

**Spectroscopic Studies.** To determine if the flavin cofactor was covalently linked to the protein in the H466Q variant, the enzyme at a concentration of 25 $\mu$M was denatured with 50% acetonitrile in 20 mM sodium phosphate (pH 7.0). After centrifugation for 15 min at 14000*g* to remove denatured protein, the UV−visible absorption spectrum of the supernatant was determined. The extinction coefficient of the flavin bound to the H466Q enzyme was determined in 20 mM sodium phosphate (pH 7.0) after denaturation of the enzyme with 4 M urea at 40 °C for 1.5 h, based upon the $\varepsilon_{450}$ value of 11.3 $mM^{-1}$ $cm^{-1}$ for free FAD and the previously published method by Whitby et al.[29] For spectroscopic studies, the enzymes were prepared fresh just prior to being used by gel filtration through PD-10 desalting columns (General Electric, Fairfield, CT). UV−visible absorption spectra were recorded in 20 mM Tris-HCl (pH 8.0) at 25 °C, using an Agilent Technologies diode-array model HP 8453 spectrophotometer. Fluorescence emission spectra were recorded in 20 mM sodium phosphate (pH 7.0) at 15 °C, with a Shimadzu model RF-5301 PC spectrofluorometer using a 1 cm path length quartz cuvette. Protein and flavin excitation wavelengths were initially determined through excitation scanning at 3 nm/min upon setting the emission wavelength to 340 and 520 nm, respectively. All fluorescence spectra were corrected with the corresponding blanks for any absorption of the buffer and Rayleigh and Raman scatterings. For protein fluorescence, the sample at a concentration of 0.8 $\mu$M was excited at 286 nm and the emission scan was determined from 310 to 400 nm; for flavin fluorescence, the sample at an FAD concentration of 3.5 $\mu$M was excited at 469 nm (454 nm for the wild-type enzyme) and the emission scan was determined from 475 to 600 nm. Circular dichroic spectra were acquired in 20 mM sodium phosphate (pH 7.0) at 25 °C, at concentrations of protein of 0.1 and 21 $\mu$M for the far- and near-UV regions, respectively, and a concentration of flavin of 23 $\mu$M for the visible region. Using a 1 cm path length quartz cuvette, five scans were taken at a rate of 50 nm/min and corrected with their corresponding blanks to remove background interference from the buffer, and the resulting average was considered. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance I 600 NMR spectrometer equipped with a 5 mm QXI probe head. The protein samples were prepared in 20 mM sodium phosphate and 10% $D_2O$ (pH 7.0) at 25 °C, with a final protein concentration of 0.1 mM. A gradient watergate W5 pulse sequence with a 75 $\mu$s delay was used to record 1024 scans for each protein at 25 °C.[30]

**Enzyme Assays.** Enzymatic activities of the H466Q and H351Q enzymes were measured in 50 mM potassium phosphate (pH 7.0) by monitoring the initial rates of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instruments, Inc., Norfolk, England) at 25 °C. The apparent steady-state kinetic parameters of the H351Q enzyme were determined by varying the concentration of choline between 20 and 120 mM and keeping the oxygen concentration constant as atmospheric (i.e., 0.25 mM). Kinetic parameters are expressed for enzyme-bound flavin, using the extinction coefficient of 11400 $M^{-1}$ $s^{-1}$ previously determined for the wild-type enzyme.[a,26] The Michaelis−Menten equation for one-substrate kinetics was used.

Anaerobic flavin reduction of the H466Q enzyme was monitored in 20 mM sodium pyrophosphate (pH 8.0−10.0) at 25 °C, using a photodiode array UV−visible spectrophotometer and an anaerobic cuvette equipped with two side arms. The anaerobic cuvette contained the H466Q (20−25 $\mu$M in flavin content) and glucose oxidase (final concentration of 0.5 $\mu$M) enzymes in the belly of the anaerobic cuvette, whereas 75 (or 60) mM choline was contained in one side arm and 7.5 mM glucose in the other. The cuvette apparatus was made anaerobic by a 20-cycle treatment of flushing with oxygen-free argon and gas removal by applying vacuum. Once the cycles had been completed, the enzymes were mixed with glucose for 30 min to ensure complete scavenging of oxygen traces prior to initiating the reaction by mixing with choline. The initial rates thus determined with 60 and 75 mM choline were similar, consistent with being a good approximation of the rate constants of flavin reduction at a saturating choline concentration.

## RESULTS

**Purification of the H351Q and H466Q Enzymes.** The H351Q and H466Q enzymes were expressed in recombinant form and purified to high levels as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis with the protocol used for the wild-type enzyme.[26,31] The enzyme-bound flavins in both variants were present throughout the purification procedure as a mixture of oxidized and air-stable anionic flavosemiquinone, requiring dialysis at pH 6.0 to obtain fully oxidized enzymes, as previously reported for wild-type choline oxidase.[31] The absorption maxima in the visible region of the oxidized enzyme variants were centered at 376 and 455 nm for the H351Q enzyme and 361 and 453 nm for the H466Q enzyme.[31] For comparison, wild-type choline oxidase has maxima at 373 and 454 nm. In the H466Q enzyme, the flavin was noncovalently attached to the protein, as it could be extracted upon treatment with acetonitrile and centrifugation to remove denatured protein. The pelleted protein did not contain any flavin, with 100% of the released flavin recovered in the supernatant based on spectral observations (data not shown). This agrees well with previous data showing that replacement of H466 with aspartate or alanine results in ≥75% of the flavin being noncovalently associated with the protein.[15,22] In contrast, the flavin in wild-type choline oxidase and several other variants characterized thus far is covalently attached to H99.[5,8,12−14,32,33] Furthermore, the extinction coefficient of the H466Q variant was determined to


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be 10.4 $mM^{-1}$ $cm^{-1}$ with a $\lambda_{max}$ of 453 nm. This value was lower than the wild-type value of 11.4 $mM^{-1}$ $cm^{-1}$, which is consistent with an altered flavin microenvironment.[26]

The apparent steady-state kinetic parameters for the enzyme variants in which H351 or H466 was replaced with glutamine were determined in atmospheric oxygen (i.e., 0.25 mM) with choline as the substrate at pH 7.0 and 25 °C by measuring initial rates of oxygen consumption. With the H351Q enzyme, the $^{app}k_{cat}$ was 0.33 ± 0.02 $s^{-1}$, the $^{app}K_m$ was 67 ± 9 mM, and the $^{app}(k_{cat}/K_m)$ was 4.9 ± 0.8 $M^{-1}$ $s^{-1}$. No oxygen consumption could be detected with the H466Q enzyme with concentrations of choline as high as 100 mM and 10 μM enzyme in the assay reaction mixture. For comparison, wild-type choline oxidase is routinely assayed at an FAD-bound concentration of 0.1 μM, yielding an $^{app}k_{cat}$ of 15 ± 1 $s^{-1}$, an $^{app}K_m$ of 0.6 ± 0.1 mM, and an $^{app}(k_{cat}/K_m)$ of 25000 ± 6500 $M^{-1}$ $s^{-1}$.[31] Thus, H466 is clearly an essential residue in the active site of the enzyme, and the H466Q variant was therefore investigated further. Spectroscopic studies were then conducted with the H466Q variant and wild type for comparison to determine if the effect on enzyme activity was due to a lack of structural integrity in the variant enzyme.

**Spectroscopic Analysis of the H466Q Enzyme.** Our attempts to crystallize the H466Q enzyme and use X-ray crystallography to establish if the mutation affects the overall folding of the enzyme and the topology of the active site were not successful. Consequently, spectroscopic techniques, including circular dichroism of the protein and the flavin, protein and flavin fluorescence, and $^1$H NMR, were used to compare and contrast the biophysical properties of the H466Q and wild-type enzymes. The use of these techniques allowed for a thorough comparison of the secondary and tertiary structures to establish if the variant enzyme exhibited the same overall fold as the wild-type enzyme.

Figure 2A shows that the far-UV circular dichroic spectra of the H466Q and wild-type enzymes determined at pH 7.0 and 25 °C were practically indistinguishable from one another, indicating that the two enzymes have a similar amount of secondary structure elements. The near-UV circular dichroic spectrum of the H466Q enzyme was also very similar to that of wild-type choline oxidase (Figure 2B), demonstrating that the replacement of H466 with glutamine is not affecting the overall fold or tertiary structure. However, an inversion of polarity in the long wavelength dichroic band of the flavin around 450 nm was observed in the H466Q enzyme compared to that of the wild-type enzyme (Figure 2C). In principle, the negative dichroic band could be attributed to the change in the microenvironment surrounding the flavin arising from the glutamine replacement of H466 or the absence of a covalent linkage between the flavin C(8) methyl and H99. The visible circular dichroic spectrum of the flavin bound to the H99N variant of choline oxidase, in which the histidyl linkage at the C(8) position is absent,[5] was consequently determined, showing a positive dichroic signal in the long wavelength region with same polarity with respect to that of the wild-type enzyme and a weak positive dichroic band that is slightly blue-shifted with respect to that of the wild type (Figure 2C). Thus, inversion of polarity in the visible circular dichroic spectrum of H466Q likely arises from a change in the microenvironment surrounding the flavin ensuing from the replacement of histidine with glutamine.[34] The far- and near-UV circular dichroic spectra revealed that both enzymes are folded with the same secondary and tertiary structure. Consistent with these results, the visible circular dichroic spectra also demonstrate that the flavin is contained within the folded

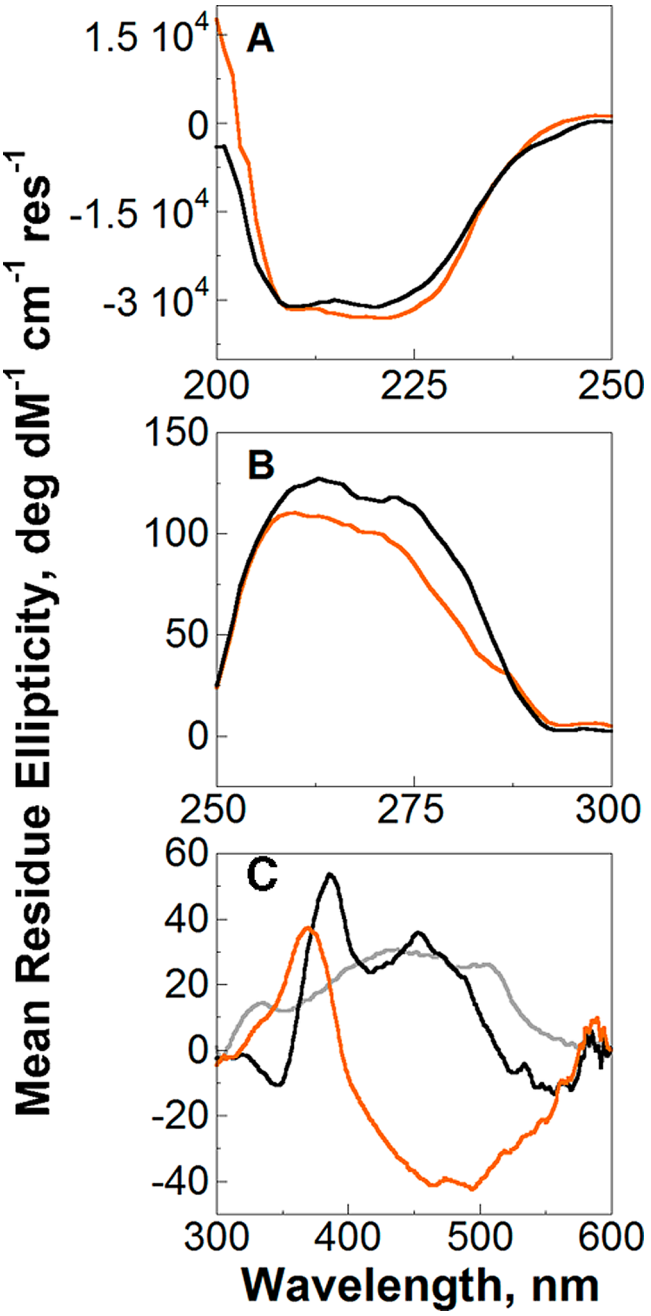


**Figure 2.** Circular dichroic spectra of H466Q (orange), H99N (gray, panel C only), and wild-type (black) enzymes, determined in 20 mM sodium phosphate (pH 7.0) at 25 °C. (A) Far-UV CD spectra were measured from 200 to 250 nm with protein concentrations of 0.1 μM. (B) Near-UV CD spectra were measured from 250 to 300 nm with protein concentrations of 21 μM. (C) Visible CD spectra were measured from 300 to 600 nm with concentrations of enzyme-bound FAD of 23 μM. Spectra were obtained by averaging five scans at 50 nm/min and were smoothed by the Means-Movement method using Jasco Spectra Analysis software.

enzyme because the induced dichroic signal at long wavelengths in the visible region of the electromagnetic spectrum reports on the protein microenvironment surrounding the isoalloxazine ring of the bound flavin and not free flavin, which lacks chiral centers in the oxidized state and yields a very weak signal.[35−37]

The protein fluorescence emission band at 342 nm was 2-fold more intense in the H466Q enzyme than in wild-type choline


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oxidase (Figure 3A). Similarly, the mutation resulted in a 5-fold increase in flavin fluorescence emission in comparison to that of

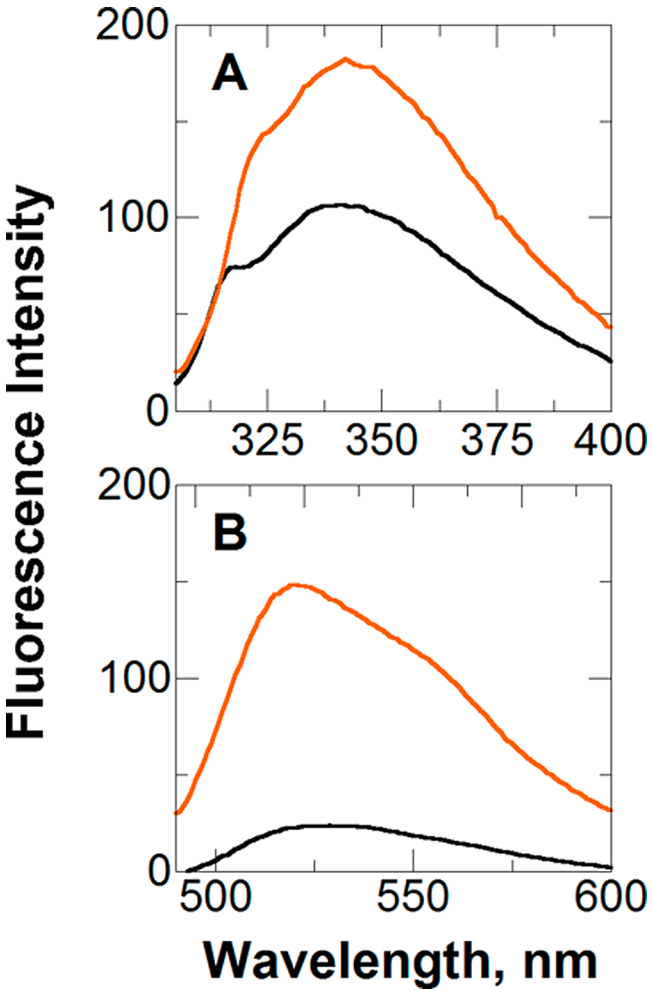


**Figure 3.** Protein and flavin fluorescence emission spectra of H466Q (orange) and wild-type (black) choline oxidase. Spectra were recorded in 20 mM sodium phosphate (pH 7.0) at 15 °C. (A) Proteins at concentrations of 0.8 $\mu$M were excited at 286 nm. (B) Enzyme-bound flavins at concentrations of 3.5 $\mu$M were excited at 454 nm (wild type) and 469 nm (H466Q).

the wild-type enzyme, with a hypsochromic shift of the band from 528 nm in the wild-type enzyme to 520 nm in the H466Q enzyme (Figure 3B). The flavin fluorescence of the wild-type and H466Q variant enzymes was quenched by >80 and 50%, respectively, in comparison to that of free FAD under the same conditions (data not shown). This agrees well with the visible circular dichroic spectra of both enzymes, in which the flavin was located within the confines of the protein.

The structural integrity of choline oxidase upon replacement of H466 with glutamine was probed also through NMR, by comparing the one-dimensional $^1$H NMR spectra of the H466Q and wild-type enzymes acquired at pH 7.0 and 25 °C in 90% $H_2O$. The NMR spectra of the two enzymes are clearly highly similar. Both wild-type and mutated enzymes exhibited good chemical dispersion, consistent with the proteins being folded (Figure 4). A closer comparison of the NMR spectra showed analogous peak patterns, line widths, and intensities in the backbone NH and aromatic regions (10.5−6 ppm) and in the aliphatic region. The resonances near 0 ppm originate from methyl groups that are shifted because of ring current effects.[38] Such signals are expected for a folded protein; a comparison shows that both enzymes have an identical fingerprint in this region. The NMR data signify similar overall folds and structure of the wild-type and variant enzymes (Figure 4).

**Anaerobic Flavin Reduction of the H466Q Enzyme.** To establish whether the enzyme-bound flavin in the H466Q enzyme could be reduced by the substrate, we incubated anaerobically the enzyme (~25 $\mu$M) with 60 mM choline and monitored the changes in absorbance at 453 nm over time. The experiment was initially conducted at pH 10.0 and 25 °C because previous steady-state and rapid reaction kinetics demonstrated that under these conditions the rate of flavin reduction is pH-independent for wild-type choline oxidase and several of its variants.[1,2,8,9,13,15,26,39] As shown in Figure 5, an ~20% decrease in absorbance at 453 nm was observed over an incubation time of 60 min, indicative of a slow reduction of the enzyme. Full reduction of the flavin could not be observed because of concerns about enzyme stability or photobleaching of the flavin over the extended reaction condition that would be required because of the impaired activity, incubation, and irradiation at 25 °C. This prevented the use of the more common exponential equations employed in rapid kinetic analysis. Instead, the method of the initial rates was used by fitting only the initial portion of the trace at 453 nm. After subtraction of the corresponding value obtained in the absence of choline (Figure 5 inset), the molar concentration of oxidized FAD reduced per unit of time was calculated and used to determine the observed rate constant for flavin reduction ($k_{obs}$). With 60 mM choline, the observed rate constant for flavin reduction ($k_{obs}$) thus calculated was $(4.6 \pm 0.1) \times 10^{-5}$ s$^{-1}$. When the reduction of the H466Q enzyme was conducted with 75 mM choline, a $k_{obs}$ value of $(4.7 \pm 0.1) \times 10^{-5}$ s$^{-1}$ was determined. This demonstrates that the enzyme is saturated at these concentrations of choline. Consequently, $\sim 5 \times 10^{-5}$ s$^{-1}$ is a good representation of the limiting rate constant for flavin reduction ($k_{red}$) at a saturating choline concentration for the H466Q enzyme. For comparison, the $k_{red}$ value at pH 10.0 and 25 °C of wild-type choline oxidase is 93 s$^{-1}$.[1] When the anaerobic reduction of the H466Q enzyme was conducted at pH 8.0 and 9.0, the $k_{red}$ values at saturating concentrations of choline were $1.3 \times 10^{-7}$ and $1.9 \times 10^{-6}$ s$^{-1}$, respectively. The decrease in the $k_{red}$ values of 2 orders of magnitude at pH 8.0 as compared to those at pH 10.0, i.e., $10^{-7}$ s$^{-1}$ versus $10^{-5}$ s$^{-1}$, is consistent with the hydride transfer reaction catalyzed by the H466Q enzyme being initiated by specific base catalysis through the hydroxide ion present in solution.[23,40]

## DISCUSSION

In the active site of choline oxidase, two histidine residues have the potential to act as a base to deprotonate choline to the alkoxide species, H351 and H466 (Figure 1). This reaction activates the alcohol substrate for the subsequent oxidation through the transfer of a hydride ion to the enzyme-bound flavin.[1,9] In previous studies, both residues were mutated to alanine, allowing us to establish mechanistic roles for H351 and H466 in substrate binding and catalysis but failing to demonstrate whether either residue is the essential base required for catalysis.[13,15] In the investigation presented here, we mutated H351 and H466 to glutamine, purified the enzyme variants, and characterized them for their ability to oxidize choline. The results demonstrated that H466 is essential in the active site of the enzyme, while replacement of H351 with glutamine impaired catalysis without completely abolishing the activity of the


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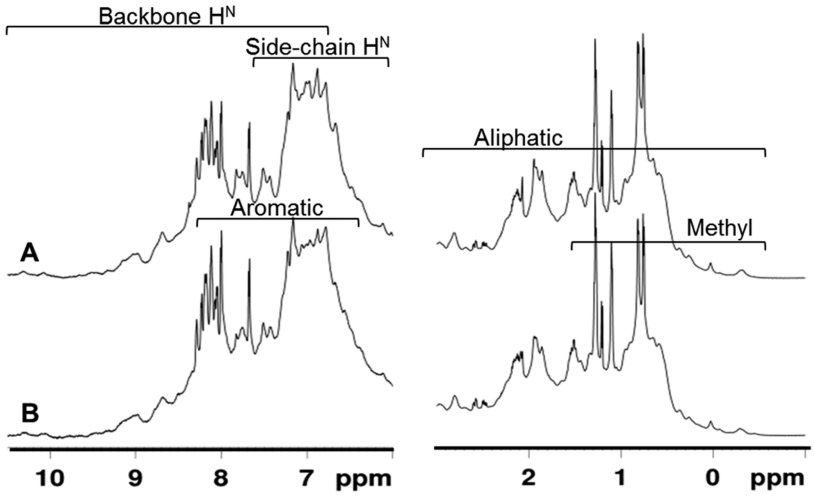



**Figure 4.** $^{1}$H NMR spectra of (A) H466Q and (B) wild-type choline oxidase in 20 mM sodium phosphate (pH 7.0) and 10% $D_2O$ at 25 °C. Both enzymes had protein concentrations of 0.1 mM.

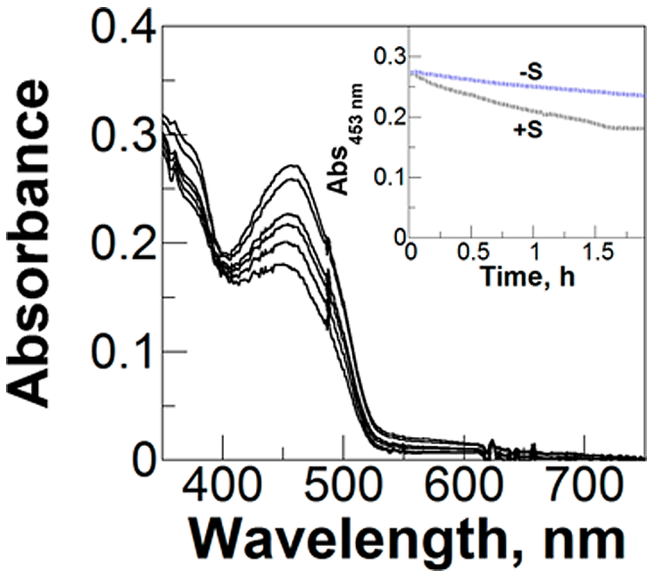


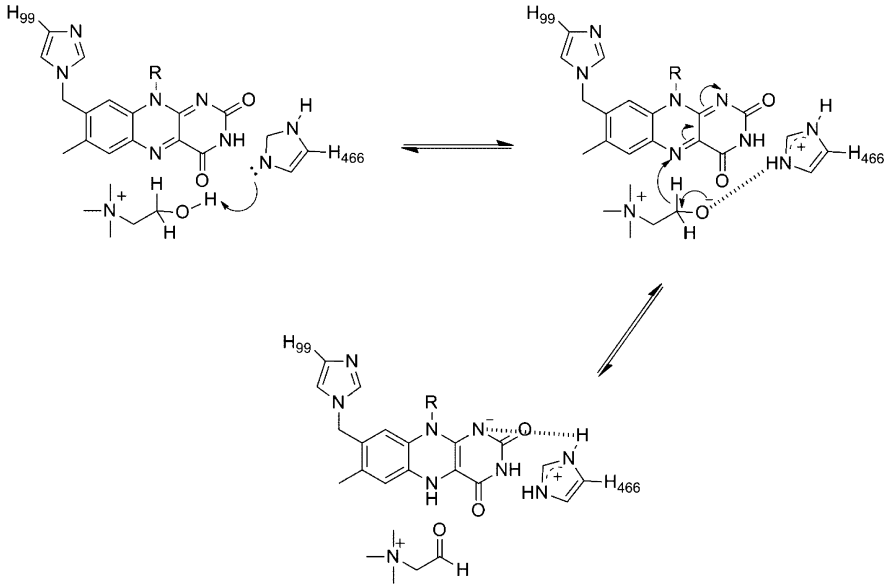

**Figure 5.** Anaerobic flavin reduction of the H466Q enzyme with 60 mM choline in 20 mM sodium pyrophosphate (pH 10.0) at 25 °C. UV–visible absorption spectra were recorded every 60 s from 350 to 750 nm. The absorbance of the flavin between 400 and 500 nm decreased over the measured time, indicating flavin reduction due to reaction with choline. In the inset, the black trace is the time course of absorbance changes at the $\lambda_{max}$ of the flavin cofactor, 453 nm, for the H466Q enzyme while the blue trace is that of a control assay in the absence of choline to account for the slow rate of photobleaching of the enzyme-bound flavin caused by intermittent light exposure over the 2 h required to acquire the data.

enzyme. This is in agreement with previous results obtained upon substitution of H351 with alanine.[13] On the basis of these findings and previous mechanistic and structural information, we conclude that H466 is the base that initiates choline oxidation in the reaction catalyzed by choline oxidase, as discussed below.

In the active site of choline oxidase, H466 is essential for catalysis. Evidence that supports this conclusion comes from both steady-state kinetics and anaerobic reduction of the H466Q enzyme with choline as the substrate. No consumption of oxygen was detected in a Clark-type oxygen electrode when the H466Q enzyme was assayed at enzyme concentrations 100-fold greater than the concentration routinely used with wild-type choline oxidase. These kinetic assays were performed with 100 mM choline, which is 160- and 1.5-fold larger than the $^{app}K_m$ values for choline determined at atmospheric oxygen with the wild-type and H351Q enzymes, respectively. Thus, the H466Q enzyme has an undetectable rate of turnover with choline using the standard kinetic assay routinely employed for choline oxidase. The lack of enzymatic activity of the H466Q enzyme was not due to the misfolded enzyme that may arise from mutating H466 to glutamine, as suggested by anaerobic flavin reduction and spectroscopic data for the H466Q enzyme. The enzyme-bound flavin was indeed reduced upon anaerobic incubation of the H466Q enzyme with choline, although with a rate constant for flavin reduction ($k_{red}$) that was 6 orders of magnitude lower than the corresponding $k_{red}$ value of the wild-type enzyme, i.e., $\sim 5 \times 10^{-5}$ $s^{-1}$ compared to 93 $s^{-1}$ at pH 10.0 and 25 °C. Under these conditions, flavin reduction is independent of pH in wild-type choline oxidase and several of its variants.[5,8,13,15] The low $k_{red}$ value determined with the H466Q enzyme agrees well with the lack of oxygen consumption observed when the enzyme is assayed using an oxygen electrode using choline as the substrate. The residual $k_{red}$ determined for the H466Q enzyme using the modified method developed in this study is clearly outside typical ranges seen for enzymatic catalysis, because it would require approximately 1 day for a single catalytic turnover to approach completion[b] at pH 10.0. Hydroxide ion with unusual basicity due to its proximity to the trimethylammonium headgroup of choline is the probable catalyst that activates choline to the alkoxide species in the active site of the H466Q enzyme, as suggested by the effect of pH on the $k_{red}$ value of the H466Q enzyme.

Independent evidence of the H466Q enzyme being folded like the wild-type enzyme comes from the circular dichroism and $^{1}$H NMR data, showing that the H466Q and wild-type enzymes had near-UV circular dichroic spectra that lacked significant differences between one another and $^{1}$H NMR spectra that did not show significant differences between the two enzymes. Furthermore, the two enzymes had far-UV dichroic spectra that were practically indistinguishable from one another. Taken together, these data are consistent with the replacement of H466 with glutamine not inducing significant protein conformational changes in the secondary structure content or overall tertiary structure of the enzyme. In this context, previous studies have

**Scheme 2. Action of H466 as the Initiator of Alcohol Oxidation and the Stabilizer of the Alkoxide Species and the Anionic Reduced Flavin**



shown that the active site of choline oxidase accommodates mutations very well, with enzyme variants showing structures that are identical to that of the wild type, as established by X-ray crystallography for the S101A[12] and V464A[33] enzymes, or with similar spectroscopic, biophysical or kinetic properties as in the case of the H99N,[5] E312D,[8,10,41] H351A,[13] V464A/T,[33] H466D/A,[15,22] and N510A/H/L[14] enzymes.

Mutation of H466 to glutamine affected the spectroscopic properties of the flavin in choline oxidase, as expected because the side chain of H466 is 3.9 Å from the flavin N(1) atom with proper orientation to participate in a H-bond with the N(1) atom of the flavin (Figure 1). Evidence supporting this conclusion comes from the comparison of the fluorescence and circular dichroic spectra of the H466Q enzyme with those of wild-type choline oxidase. The magnitude of the protein fluorescence emission band at 340 nm increased by 2-fold, while that of the flavin at 530 nm increased by 5-fold upon substitution of H466 with glutamine, consistent with a decrease in hydrophobicity around the aromatic residues responsible for protein fluorescence and flavin fluorescence in the active site of the enzyme. Consistent with the observed changes in the absorbance and extinction coefficient of the H466Q enzyme with respect to wild-type choline oxidase, an effect on the hydrophobicity of the surrounding flavin environment is also suggested by the hypsochromic shift of the emission band from 528 to 520 nm in the H466Q enzyme compared to that of the wild type.[42−44] The active site of choline oxidase contains aromatic residues, including W61, F357, Y465, and W331,[6] whose fluorescence emission is likely sensitive to the nature of the side chain at position 466 because of proximity effects. The increase in flavin fluorescence observed in the H466Q enzyme compared to that of the wild type can thus be ascribed to a diminished quenching of flavin fluorescence exerted by the side chain at position 466 of the protein with glutamine rather than histidine. The positive dichroic peak in the near-UV−visible region (350−400 nm) is comparable to that in the measured CD spectra of other flavoprotein oxidases, such D-amino acid oxidase, L-amino acid oxidase, and glucose oxidase.[45] The hypochromism and blue-shift in the near UV−visible circular dichroic spectra of the H466Q variant are consistent with the fluorescence and absorbance spectral shifts being affected by the altered active site polarity and nearby aromatic residues.[45]

Replacement of H466 with glutamine prevents flavinylation of the protein through the formation of a covalent linkage with H99, which is otherwise observed in wild-type choline oxidase and several of its variants.[5,8,12−15,26,32,33] Evidence of this conclusion comes from the analysis of the supernatant obtained through treatment of the H466Q enzyme with acetonitrile followed by centrifugation to remove denatured protein, showing that 100% of the flavin could be extracted from the enzyme. This agrees well with previous results for the H466D variant of choline oxidase, in which ∼75% of FAD was not covalently linked to the protein.[22] Curiously, when H466 was replaced with alanine, there was no effect on enzyme flavinylation,[15] although the reason is unclear and further studies are required. All the other residues previously targeted for mutagenesis in the active site of the enzyme, including S101,[12] E312,[8] H351,[13] V464,[33] and N510A/H/L,[14] retained the cofactor covalently linked to the protein. Thus, the current results obtained with the H466Q enzyme, taken together with the results previously obtained with other variants of choline oxidase, reinforce the importance in the flavinylation process of H466, whose side chain is close to the flavin N(1) and C(2) atoms (Figure 1). In trimethylamine dehydrogenase and monomeric sarcosine oxidase, the presence of a protein positive charge close to the flavin N(1) and C(2) atoms has been shown to be important in the flavinylation process.[46,47]

The results of the mutagenesis investigation of H466 presented here and in a previous study in which the residue was mutated to alanine, in conjunction with mechanistic and structural studies using X-ray crystallography of the enzyme in complex with the product of reaction glycine betaine,[6,15] establish H466 as a key residue playing multiple roles in the catalytic mechanism of choline oxidase. After formation of the enzyme−substrate complex, H466 removes the hydroxyl proton of choline with formation of an alkoxide species (Scheme 2). Previous pH and deuterium kinetic isotope effect studies using steady-state and rapid reaction kinetics are consistent with an intrinsic p$K_a$ value of 7.5 for the catalytic base in the active site of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the enzyme, which is now assigned to the side chain of H466.[1,9,26,39,48] Alcohol deprotonation triggers the subsequent hydride transfer reaction from the $C^{\alpha}$ atom of the alkoxide to the flavin N(5) atom, which is fully rate-limiting for the overall enzyme turnover and the reductive half-reaction in the wild-type enzyme.[1] Hydride transfer occurs in concerted but asynchronous fashion with the proton already residing on the side chain of H466 in the transition state, as established previously by a lack of solvent and large substrate kinetic isotope effects on the $k_{red}$ value for the wild-type enzyme and the partial rescuing of the $k_{cat}$ value in the H466A enzyme by imidazolium.[1,15] In contrast, previous solvent kinetic isotope effects on the H466A variant revealed a more rate-limiting O−H bond cleavage with a $^{D}(k_{cat}/K_{m})$ value of 2.2 compared to the value of 1 observed in the wild-type enzyme.[15] Consequently, mutation of H466 to alanine altered the mechanism from stepwise to concerted,[15] whereas mutation to glutamine completely abolished catalysis (this study). Thus, H466 fills in catalysis a dual role as the base activating the substrate for catalysis and as an electrostatic catalyst stabilizing the partial charge developing on the $C^{\alpha}$ atom of the alkoxide during substrate oxidation. Mutagenesis, kinetic, and computational studies have recently established that the residue equivalent to H466 in the active site of two other members of the glucose−methanol−choline oxidoreductase enzyme superfamily, namely, H502 in aryl-alcohol oxidase[21] and H548 in pyranose 2-oxidase,[17] also acts as a base in catalysis.

In summary, we have used mutagenesis, along with kinetic and spectroscopic approaches, to establish the identity of the catalytic base in the active site of choline oxidase. The results presented herein on a variant of choline oxidase with H466 substituted with glutamine are consistent with this residue being the base that deprotonates choline to the alkoxide species in the catalytic mechanism of the enzyme. This agrees well with the recent crystallographic structure of the enzyme in complex with glycine betaine, showing that the side chain of H466 is 3.1 Å from one of the carboxylate O atoms of the ligand (Figure 1).[6] The apparent discrepancy in the results obtained with the H466Q enzyme in this study and the H466A enzyme in a previous study is reconciled with the hydroxide ion likely occupying the space vacated by the imidazole side chain in the latter enzyme and acting as a (poor) catalyst to activate choline to alkoxide for subsequent flavin reduction through hydride transfer. In this respect, previous mechanistic studies have established that a water molecule in the active site of the enzyme is necessary for the hydration of betaine aldehyde prior to the second oxidation reaction yielding glycine betaine.[1] Therefore, the histidine residue at position 466 holds multiple functions in the reductive half-reaction of choline oxidase. It exists as an electrostatic catalyst stabilizing the negative charges present on the reduced flavin and alkoxide intermediate and acts as the catalytic base that activates choline. Multiple amino acid substitutions had to be evaluated to elucidate the roles of this important residue in the mechanism of choline oxidase.

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### Notes

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## ADDITIONAL NOTES

[a]An inaccuracy of up to 25% may have resulted in the determination of the apparent steady-state kinetic parameters of the H351Q enzyme by using the extinction coefficient of the flavin bound to the wild-type enzyme. This does not alter the main conclusion that is drawn in this study that the mutation results in an enzyme that retains the ability to oxidize choline. Because the H351Q enzyme was not investigated further and the kinetic parameters determined at an atmospheric oxygen level cannot be used to draw mechanistic conclusions, we did not attempt a more accurate determination of the concentration of enzyme-bound flavin based on the extinction coefficient of the mutant enzyme.

[b]A first-order process of flavin reduction with an apparent rate constant of $10^{-5}$ $s^{-1}$ has a half-life of 13860 s, equivalent to 3.85 h. Thus, 98.5% completion, which is achieved in six half-lives, would require 23.1 h. In comparison, assuming no loss of activity, the wild-type enzyme would have completed 7.7 million turnovers in the same time span of 23 h as H466Q would only have completed a single turnover.

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