Hydride Transfer Made Easy in the Reaction of Alcohol Oxidation Catalyzed by Flavin-dependent Oxidases[†,‡]

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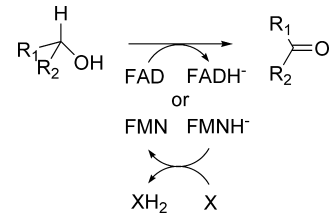
ABSTRACT: Choline oxidase (E.C. 1.1.3.17; choline-oxygen 1-oxidoreductase) catalyzes the two-step, four-electron oxidation of choline to glycine betaine with betaine aldehyde as enzyme-associated intermediate and molecular oxygen as final electron acceptor. Biochemical, structural, and mechanistic studies on the wild-type and a number of mutant forms of choline oxidase from *Arthrobacter globiformis* have recently been carried out, allowing for the delineation at molecular and atomic levels of the mechanism of alcohol oxidation catalyzed by the enzyme. First, the alcohol substrate is activated to its alkoxide species by the removal of the hydroxyl proton in the enzyme−substrate complex. The resulting activated alkoxide is correctly positioned for catalysis through electrostatic and hydrogen bonding interactions with a number of active site residues. After substrate activation and correct positioning are attained, alcohol oxidation occurs in a highly preorganized enzyme−substrate complex through quantum mechanical transfer of a hydride ion from the α-carbon of the chelated, alkoxide species to the N(5) atom of the enzyme-bound flavin. This mechanism in its essence is shared by another class of alcohol oxidizing enzymes that utilize a catalytic zinc to stabilize an alkoxide intermediate and $NAD(P)^+$ as the organic cofactor that accepts the hydride ion, whose paradigm example is alcohol dehydrogenase. It will be interesting to experimentally evaluate the attractive hypothesis of whether the mechanism of choline oxidase can be extended to other flavin-dependent enzymes as well as enzymes that utilize cofactors other than flavins in the oxidation of alcohols.

The oxidation of alcohols to aldehydes or ketones is catalyzed by a large number of flavin-dependent enzymes, including among others choline oxidase (*1*), choline dehydrogenase (*2*), methanol oxidase (*3*), glucose oxidase (*4*), cholesterol oxidase (*5*), pyranose 2-oxidase (*6*), cellobiose dehydrogenase (*7*), vanillyl-alcohol oxidase (*8*), alditol oxidase (*9*), glycolate oxidase (*10*), lactate oxidase (*11*), and flavocytochrome b2 (*12*). These enzymes catalyze the transfer of a hydride equivalent (i.e., 2 e^- and 1 H^+) from the carbon in position α of an alcohol substrate to an FAD or FMN cofactor that is tightly or covalently bound to the protein moiety, as illustrated in Scheme 1. Enzyme turnover is then completed with the oxidation of the enzyme-bound flavin via the transfer of electrons to a specific electron acceptor (*13*), which is generally molecular oxygen in the flavoprotein oxidases, an iron−sulfur cluster or pyridine dinucleotide in the class of the dehydrogenases, or a heme group in flavocytochromes. Several proposals have been put forth over the past decades to describe the chemical mechanisms for alcohol oxidation catalyzed by flavin-dependent enzymes, including oxygen and carbon-based radical mechanisms,

Scheme 1: Reaction of Alcohol Oxidation Catalyzed by Flavin−dependent Enzymes



stabilization of carbanion intermediates, and hydride ion transfer reactions (for recent reviews on the topic, see refs *14* and *15*). Since the publication of the most recent review on the topic in 2004 (*15*), a novel flavin-dependent alcohol oxidase, choline oxidase, has been the object of intensive studies aimed at the elucidation of the mechanism of alcohol oxidation. In this Current Topic, the lessons that have been learned on the reaction of alcohol oxidation catalyzed by choline oxidase from *Arthrobacter globiformis* using biochemical (*16*−*19*), structural (*20*), site-directed mutagenic (*1, 18, 20*−*22*), and mechanistic (*23*−*30*) approaches will be presented. The results obtained with choline oxidase will be compared to those available for other flavin-dependent enzymes that oxidize alcohols. Finally, the similarities in the catalytic strategies for alcohol oxidation used by the flavin-dependent choline oxidase and the zinc-dependent alcohol dehydrogenase that uses $NAD(P)^+$ as electron acceptor will be discussed, raising the interesting question of whether there exists a common strategy for the oxidation of alcohols to

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[‡] I dedicate this review article to the dear memory of Professor Bruno Curti (1931−2008), who has been and continues to be a source of professional and personal inspiration as a mentor, colleague and example.

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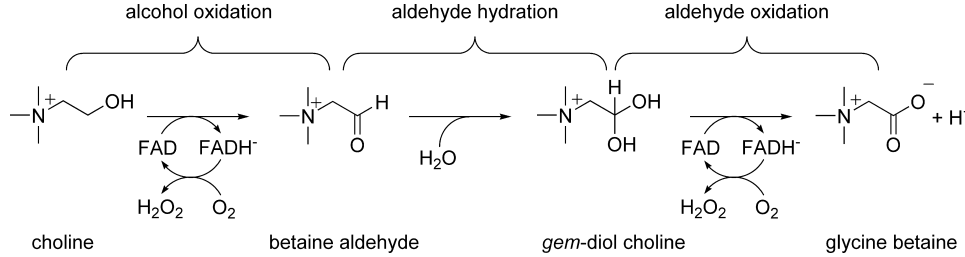
Scheme 2: Two-step, Four-Electron Oxidation of Choline Catalyzed by Choline Oxidase



the corresponding carbonyl compounds catalyzed by enzymes with organic cofactors.

General Reaction Catalyzed by Choline Oxidase. Choline oxidase (E.C. 1.1.3.17; choline-oxygen 1-oxidoreductase) catalyzes the four-electron, two-step oxidation of choline to glycine betaine with the formation of an aldehyde intermediate (Scheme 2) (*24, 31*). The first oxidation reaction involves the transfer of a hydride ion from the alcohol substrate to the enzyme-bound flavin, yielding betaine aldehyde and anionic flavin hydroquinone (*27*). This reaction is then followed by an oxidative half-reaction in which the flavin hydroquinone is oxidized with concomitant reduction of molecular oxygen to hydrogen peroxide (*31*). Kinetic and mechanistic studies showed that the aldehyde intermediate predominantly stays bound at the active site of the enzyme during turnover of the enzyme with choline (*24, 27*), where it is rapidly hydrated to the *gem*-diol form (Scheme 2) (*28*). The second oxidation reaction involves the transfer of a hydride equivalent from the hydrated aldehyde to the flavin and the subsequent oxidation of the ensuing reduced flavin by molecular oxygen (*28*). The observation that the *gem*-diol form of the aldehyde acts as substrate in the oxidation reaction (*28*) strongly suggests that the enzyme may use similar strategies for oxidation of both the alcohol substrate and the aldehyde intermediate.

Biophysical Properties of Choline Oxidase. Both X-ray crystallographic (*20*) and biochemical studies in solution (*17*) support the notion that choline oxidase exists as a homodimer of 120 kDa, with each subunit containing FAD covalently linked through its C(8) methyl group to the N(3) atom of His99 (*20*). The dimer interface includes two sets of six identical intersubunit contacts between oppositely charged residues clustered on the outer edges of the interface, with minimal close contacts of the central portions of the dimer interface between subunits (Figure 1) (*20*). The active sites are formed by identical cavities of ~125 Å^3 facing the *re* face of the flavin in each subunit, which are completely secluded from the exterior of the protein by a long loop region composed of residues 64−95. The central portions of the loops extend into the bulk solvent, suggesting that residues 74 to 85 in each subunit may form a lid that allows entry of the alcohol substrate and exit of the reaction product to and from the active sites. Similar loop regions are present also in other flavoenzymes that oxidize alcohols, such as cholesterol oxidase (*32, 33*), pyranose 2-oxidase (*34*−*36*), glucose oxidase (*37*−*40*), and cellobiose dehydrogenase (*41*). However, the molecular mechanisms of entry of the substrates and exit of the products into and from the active sites of these enzymes and of choline oxidase are important questions that wait to be addressed.

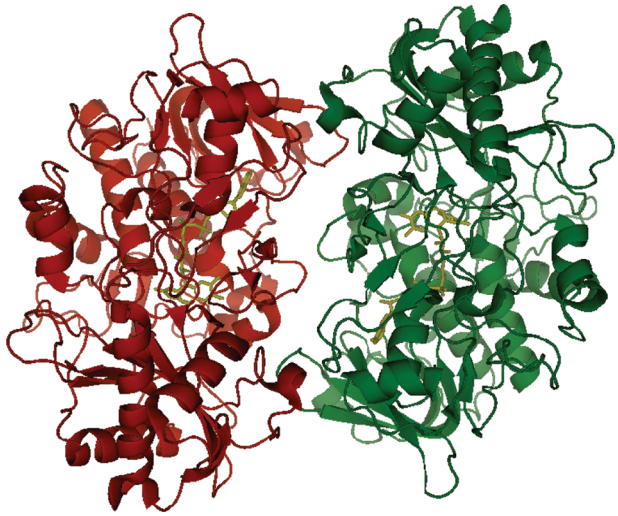


FIGURE 1: Three-dimensional structure of choline oxidase refined to a resolution of 1.86 Å (Protein Data Bank 2jbv). The dimeric structure of the enzyme is shown with the two identical subunits colored in red and green. FAD molecules in each subunit are rendered in yellow.

The enzyme-bound oxidized flavin of choline oxidase shows typical UV−visible absorbance peaks at 359 and 452 nm at pH 8.0 (with ε_{452} of 11,400 M^{-1}cm^{-1}) and emits light at 530 nm when excited at 452 nm (*16*). Anaerobic substrate reduction of the enzyme yields an anionic hydroquinone species with a well-defined absorbance maximum at 356 nm (Figure 2) (*16*). This reduced species is stabilized at all pH values between 6.0 and 10.0 (*18*), suggesting that the reduced flavin is anionic in this pH range. The enzyme has a high affinity for sulfite, with a K_d of ~50 μM at pH 7.0 and 15 °C (*16*), and stabilizes the anionic species of the flavin semiquinone upon anaerobic reduction with dithionite (Figure 2) (*16, 18*). In agreement with these observations, which all point to stabilization of a negative charge on the N(1)−C(2) atoms of the one- and two-electron reduced flavin (*42*), the X-ray crystallographic structure of the enzyme defined to a resolution of 1.86 Å shows the N-terminal end of a long α-helix and the side chain of His466 less than 4 Å away from the N(1)−C(2) atoms of FAD (*20*). Consistent with an involvement of His466 in the stabilization of a negatively charged flavin, anaerobic reduction of an enzyme variant in which His466 is replaced with aspartate results in the stabilization of a neutral hydroquinone, rather than the anionic species, with lack of formation of an intermediate flavosemiquinone (*18*).

At pH 8.0, the anionic flavosemiquinone of choline oxidase is unusually insensitive to both molecular oxygen and artificial electron acceptors (*16*). In contrast, at pH 6.0 and 4 °C the anionic flavosemiquinone is slowly oxidized under aerobic conditions to the fully oxidized state, possibly through a pH-dependent conformational change of the

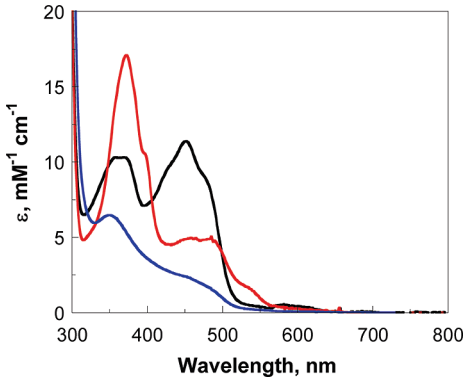


FIGURE 2: UV−visible absorbance spectra of choline oxidase in oxidized (black), semiquinone (red), and hydroquinone (blue) states. Spectra were recorded in 20 mM Tris-Cl at pH 8.0. The enzyme-bound semiquinone was obtained upon anaerobic titration of the enzyme with dithionite; the hydroquinone was obtained upon anaerobic reduction of the enzyme with 4.5 mM betaine aldehyde.

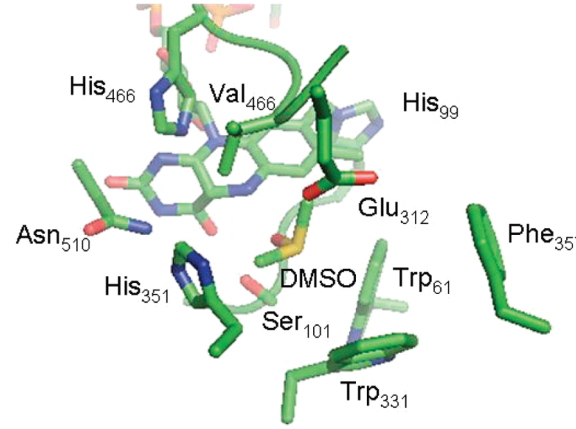


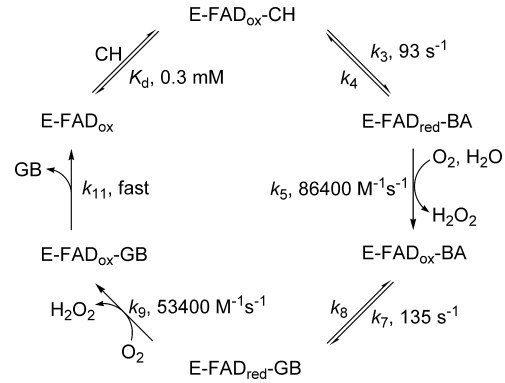
FIGURE 3: Active site of choline oxidase (PDB 2jbv). Selected amino acid residues are shown along with DMSO, an additive used in the crystallization process. Note the significant distortion of the flavin ring at the C(4a) atom, which is due to the presence of a C(4a) flavin adduct (not shown here), whose identity is currently under investigation.

enzyme that allows reaction of the flavosemiquinone with oxygen (*16*). Notwithstanding the interesting question of what provides such an unusual lack of reactivity, from a mechanistic standpoint the anionic flavosemiquinone of choline oxidase does not directly participate in the reductive half-reaction of alcohol oxidation as indicated by both UV−visible absorbance studies on the enzyme under turnover with choline, and pre steady-state anaerobic reductions of the enzyme with choline or *gem*-diol betaine aldehyde as substrate (*16, 27*). A similar lack of reactivity of an anionic flavosemiquinone toward molecular oxygen was previously reported for methanol oxidase (*43*), for which a mechanism for the unusual lack of reactivity was not elucidated.

Choline oxidase in the free form devoid of ligands shows midpoint reduction−oxidation potentials for the enzyme-bound FAD that are among the highest that have been reported, with $E_{ox/sq,7}$ and $E_{sq/red,7}$ values of +211 ± 2 mV and −65 ± 2 mV for the oxidized/semiquinone and semiquinone/reduced couples at pH 7.0 (*18*). Upon binding of the reaction product, glycine betaine, in the active site the enzyme loses the ability to thermodynamically stabilize the semiquinone, showing a midpoint reduction−oxidation potential $E_{ox/red,7}$ value of +132 ± 1 mV for the oxidized/reduced couple (*21*). The elevated values for the midpoint reduction−oxidation potentials in choline oxidase are due in part to the covalent linkage of the flavin to the protein moiety, which contributes ~100 mV (*29*), and in part to the presence of the positive charge provided by the side chain of His466 in proximity of the N(1)−C(2) atoms of the flavin, as suggested by site-directed mutagenesis studies in which His466 is replaced with alanine or with aspartate (*18, 21*). Future investigations will be required to further dissect the contributions of other amino acid residues that surround the flavin to the elevation of the midpoint reduction−oxidation potentials of choline oxidase, such as, for example, Asn100, Ser101, Val464, or Asn510 (Figure 3).

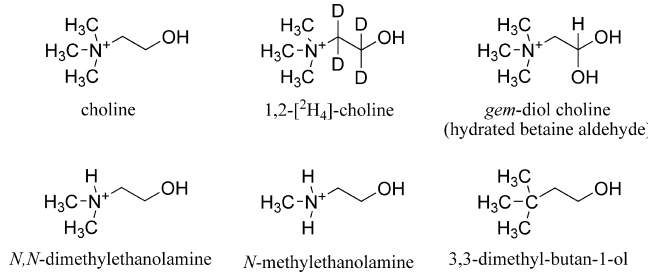
Steady-State Kinetic Mechanism. The minimal steady-state kinetic mechanism of choline oxidase with choline as substrate is shown in Scheme 3. This mechanism is valid for pH values between 5.0 and 10.0 (*16, 24, 26*), indicating that the order of the kinetic steps involving substrate and product binding to the enzyme is not affected by pH. Substitution of several active site residues by site-directed mutagenesis, such as Glu312 (*20*), His351 (*1*), Val464 (*22*), His466 (*21*), as well as His99 or Ser101 (Sharonda Cowins, Osbourne Quaye, Hongling Yuan, and Giovanni Gadda, unpublished observations), as well as the use of substrate analogues such as 1,2-[$^2H_4$]-choline, *gem*-diol choline (i.e., hydrated betaine aldehyde), *N,N*-dimethylethanolamine, *N*-methylethanolamine, and 3,3-dimethyl-butan-1-ol (Chart 1) (*23, 25, 27, 28*) do not change the order of substrate and product binding to the enzyme. Both in the wild-type and a number of active site mutagenized enzymes, the overall turnover (k_{cat}) with

Scheme 3: Minimal Steady-State Kinetic Mechanism of Choline Oxidase[a]

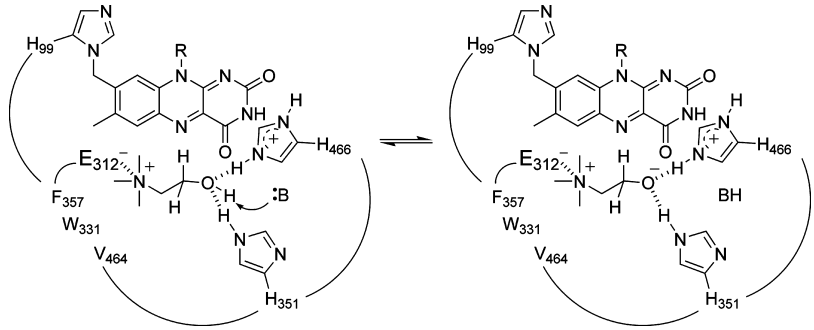


[a] Values for individual rate constants are for pH 10.0 (*27*), where kinetic steps are pH-independent (*16*). E, enzyme; FAD_{ox}, oxidized flavin; FAD_{red}, reduced flavin; CH, choline; BA, betaine aldehyde; GB, glycine betaine.

Chart 1



Scheme 4: Abstraction of the Hydroxyl Proton of Choline that Initiates the Alcohol Oxidation Reaction in Choline Oxidase[a]



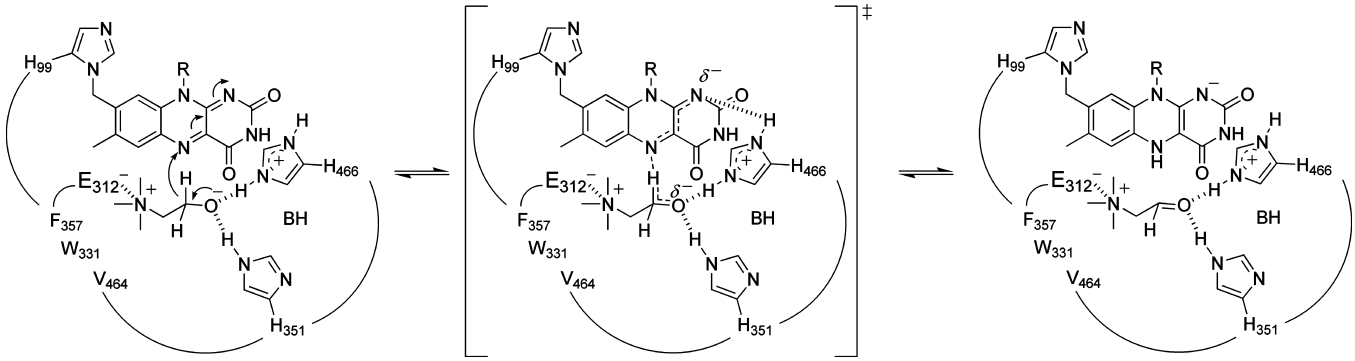
[a] The dashed lines represent electrostatic or hydrogen bonds between active site residues and the alcohol substrate; B is an unidentified catalytic base.

choline or 1,2-[$^2H_4$]-choline as substrate is limited by the steps in which hydride ions are transferred from the alkoxide choline and the *gem*-diol choline intermediate to the enzyme-bound flavin (k_3 and k_7 in Scheme 3), as indicated by direct measurement of the rate constants for flavin reduction using anaerobic stopped-flow spectrophotometry (k_{red}) and steady-state kinetics measurements (*1, 20, 21, 27*).

Activation of the Alcohol Substrate in Catalysis. Catalysis is initiated in the enzyme−substrate complex with the abstraction of the hydroxyl proton of choline by a catalytic base with pK_a of ~7.5 (Scheme 4), as suggested by pH and kinetic isotope effects studies on the k_{cat}/K_m and k_{cat} values with choline and a number of substrate analogues with the wild-type form of choline oxidase (*16, 23, 25*−*28*). Inhibition studies with glycine betaine, as well as substrate kinetic isotope effects with 1,2-[$^2H_4$]-choline are consistent with the pK_a of 7.5 being a thermodynamic value (*16, 26, 27*). The formation of the alkoxide species that results from the proton abstraction step is kinetically fast and mechanistically decoupled from the subsequent, slow transfer of the hydride ion from the α-carbon of the activated alkoxide species to the N(5) atom of the flavin, as suggested by solvent, substrate, and multiple kinetic isotope effects on the steady state kinetic parameter k_{cat}/K_m with choline as substrate (Scheme 4) (*27*). Both Val464 and His466 contribute to the activation of the alcohol substrate, as suggested by mutagenesis studies showing that their substitution results in the hydroxyl proton abstraction occurring with a rate constant that is comparable to that of the hydride transfer reaction (*21, 22*). In the enzyme where His466 is replaced with alanine, the effect is due to the lack of the positively charged side chain of His466, which electrostatically stabilize the negatively charged alkoxide species (*21, 22*). In the enzymes where Val464 is replaced with threonine or alanine, the effect is likely due to the incorrect positioning of His466 as a result of the adjacent less hydrophobic residues (*22*). Among flavin-dependent enzymes other than choline oxidase, the transient formation of an activated alkoxide species in catalysis was shown in an elegant study on methanol oxidase that combined the use of substrate analogues and kinetic isotope effects (*3*). With flavocytochrome b2 (*44*), glucose oxidase (*45*), and cholesterol oxidase (*46, 47*), steady-state kinetic determinations showed solvent kinetic isotope effects of unity and substrate kinetic isotope effects between 2 and 3 on the k_{cat}/K_m values. These observations, although not conclusive due to the kinetic complexity of these enzymes, are at least consistent with the model proposed for choline oxidase, where the fast deprotonation of the hydroxyl group of the substrate yields an activated, alkoxide species from which a hydride ion is transferred to the isoalloxazine moiety of the flavin.

In choline oxidase, two histidine residues are suitably located in the active site cavity of the enzyme with the potential to activate choline during turnover of the enzyme, namely, His351 and His466 (Figure 3) (*20*). Replacement of either residue with alanine results in enzyme variants with k_{cat} and k_{cat}/K_m values for choline that are between 20- and 60-fold lower than the values for the wild-type enzyme in the pH independent region, i.e., at pH 10 (*1, 21*). Interestingly, the presence of an unprotonated group that acts as catalytic base is still observed in the pH profiles of the k_{cat} and k_{cat}/K_m values with both the His351Ala and His466Ala enzymes, although with thermodynamic pK_a values increased to 8.0 and 9.0, respectively (cfr. 7.5 for the wild-type enzyme) (*1, 21*). While the increase in the pK_a values for the catalytic base is consistent with both His351 and His466 contributing to the overall polarity of the active site for efficient alcohol deprotonation, the presence of an unprotonated group in catalysis in the histidine to alanine single enzyme variants suggests that neither histidine residue is solely responsible for the activation of the substrate. In the case of His466, this conclusion is independently supported by kinetic data showing that the enzymatic activity of the His466Ala enzyme can be partially rescued in the presence of imidazolium at pH <7.0 but not with imidazole at pH ≥7.0 (*21*). Since no other ionizable groups are present in the active site of the enzyme (*20*), an attractive possibility is that either one of the two histidines, possibly His351, 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 mutagenized enzyme. Alternatively, the acidification of the substrate hydroxyl group, perhaps associated to the rapid loss of the proton to the solvent, may occur through electrostatic and hydrogen bonding interactions established with multiple residues in the active site of the enzyme. In choline oxidase, these residues may be Ser101, His351, His466, and the C(4) oxygen atom of the flavin cofactor (Figure 3). A qualitatively similar mechanism was recently proposed for cholesterol oxidase by Lario, Sampson, and Vrielink as a possible way by which that enzyme can activate the substrate for the oxidation reaction (*48*). In that

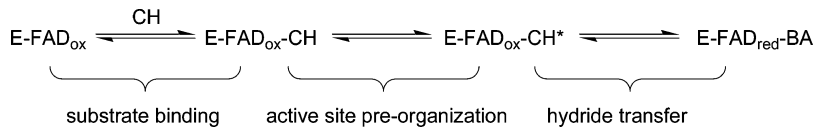
Scheme 5: Hydride Ion Transfer Reaction from the α-Carbon of Activated Alkoxide Choline to the N(5) Atom of the Enzyme-Bound Flavin of Choline Oxidase



case, the decrease in the pK_a value of the hydroxyl group of the steroid substrate would be provided by the side chain of Glu361 and the lone pair electrons on the sulfur atom of Met122 and the C(4) oxygen atom of the flavin cofactor. Studies on double mutant variants of choline oxidase in which both His351 and His466 are replaced with other residues are currently in progress to address this issue. In a number of flavin-dependent enzymes that oxidize alcohols other than choline oxidase, histidine residues have been proposed to be responsible for the activation of the substrate in catalysis, including glucose oxidase (*37*−*40*), pyranose 2-oxidase (*34*−*36, 49*), cellobiose dehydrogenase (*41, 50*), glycolate oxidase (*10*), lactate monooxygenase (*51*), and flavocytochrome b2 (*12, 52, 53*). While the evidence in support of the conclusion is either structural or from site-directed mutagenesis, a pH-dependence analysis of the kinetic parameters of mutant enzymes where the putative active site base is selectively replaced by other amino acid was carried out only in the case of activity rescuing experiments with mutant variants of mandelate dehydrogenase where His274 was selectively replaced by alanine or glycine (*54*). Thus, it appears that in the entire class of enzymes evidence for a single histidine residue being responsible for the activation of the alcohol substrate is not conclusive.

Stabilization of the Alkoxide Species. Stabilization of the transient alkoxide intermediate in the active site of choline oxidase is achieved through electrostatic and hydrogen bonding interactions of the trimethylammonium headgroup and the alkoxide oxygen atom with side chains of amino acids in the active site of the enzyme. Mechanistic data for mutant forms of choline oxidase in which Glu312 is replaced with aspartate, glutamine, or alanine (*20*), and His466 is replaced with aspartate or alanine (*18, 21*) strongly suggest that these active site residues play crucial roles for the stabilization of the alkoxide intermediate that is formed in catalysis. The negatively charged side chain of Glu312 interacts electrostatically with the positively charged trimethylammonium moiety of the organic substrate and, presumably, with any transition state, organic intermediate, and product of the enzymatic reaction. Indeed, replacement of Glu312 with glutamine results in a 500-fold increase in the K_d value for choline (i.e., from 0.3 mM to ≥150 mM), as determined using rapid kinetics techniques (*20*). This corresponds to an energetic contribution of ∼15 kJ/mol for the electrostatic interaction of Glu312 with the positively charged trimethylammonium group of choline, in good agreement with the estimate of ∼13 kJ/mol that was independently determined from mechanistic studies with 3,3-dimethylbutan-1-ol, a choline analogue lacking the positive charge, (Chart 1), as substrate for the wild-type enzyme (*29*). Kinetic, spectroscopic, and biochemical studies on the enzyme variant in which His-466 is replaced with alanine strongly support the notion that His466 is protonated during the reductive half-reaction in which choline is oxidized to betaine aldehyde (*21*). Mechanistic data further suggest that the protonated histidine contributes to the electrostatic stabilization of the alkoxide intermediate and the transition state for the oxidation of choline to betaine aldehyde (Scheme 5) (*21*). Replacement of His466 with alanine indeed results in the cleavages of the OH and CH bonds being concerted rather than uncoupled as in the wild-type enzyme, as suggested by solvent and substrate kinetic isotope effects (*21*). The imidazole side chain of His351 also contributes to stabilization of the transient alkoxide species by acting as a hydrogen bond donor to the hydroxyl oxygen atom of both the alcohol substrate and the transition state, as illustrated in Schemes 4−5. Indeed, replacement of His351 with alanine has a dual effect on both the binding affinity of the enzyme for the substrate, with the K_d value increasing by 1 order of magnitude with respect to the wild-type enzyme (i.e., from 0.3 mM to 2.6 mM) (*1*), and the rate constant for anaerobic flavin reduction, with the k_{red} value decreasing 75-fold from 93 s^{-1} to 1.2 s^{-1} (*1*). Thus, His351 plays important roles for both substrate binding and the hydride ion transfer reaction catalyzed by choline oxidase. Mutagenesis studies further suggest that the hydrophobic side chain of Val464 also contributes indirectly to the stabilization of the alkoxide species through its interaction with the side chain of His466, which is required for the correct positioning of the initial alcohol substrate relative to the positive charge that interacts with the alkoxide species (*22*). Although no direct biochemical evidence has yet become available, the side chain of Ser101 appears to be suitably located in the active site of the enzyme to also contribute to the stabilization of the alkoxide oxygen atom of the activated substrate (Figure 3) (*20*). A similar stabilization of an activated, alkoxide species in catalysis was also shown for flavocytochrome b2, where an active site tyrosine was proposed to hydrogen bond the alkoxide oxygen of the activated alcohol, as suggested by kinetic isotope effects on an enzyme variant with Tyr254 substituted with phenylalanine (*55*). As for the case of choline oxidase, lack of stabilization of the alkoxide species in the Tyr254 to phenylalanine enzyme variant results in a hydride transfer reaction that is concerted with the deprotonation reaction

Scheme 6: Preorganization of the Activated Enzyme−Substrate Michaelis Complex in Choline Oxidase[a]



[a] E, enzyme; FAD_{ox}, oxidized flavin; FAD_{red}, reduced flavin; CH, choline; BA, betaine aldehyde.

required to activate the alcohol substrate, as indicated by multiple kinetic isotope effects studies (*55*).

Hydride Ion Transfer. The hydride ion transfer reaction in which choline is oxidized to betaine aldehyde by choline oxidase occurs via a quantum tunneling mechanism within a preorganized enzyme−substrate complex, where minimal independent movement of the alkoxide α-carbon acting as a hydride donor with respect to the N(5) atom of the flavin cofactor acting as a hydride acceptor is permitted (Scheme 5). This conclusion is supported by mechanistic data addressing the effect of temperature on both the second-order rate constants k_{cat}/K_m for the reaction of choline oxidase with choline and 1,2-[$^2H_4$]-choline, and the associated kinetic isotope effects, $^D(k_{cat}/K_m)$ (*26*). At saturating concentrations of oxygen, where the hydride transfer reaction is practically irreversible due to $k_4/k_5[O_2]$ ratio in Scheme 3 being negligible, the $^D(k_{cat}/K_m)$ value is 10.6 ± 0.6 and is temperature independent between 15 and 40 °C (*26*). The corresponding isotope effect on the Eyring preexponential factors (A_H'/A_D') is significantly larger than unity (14 ± 3) (*26*). Furthermore, the enthalpies of activation ($\Delta H^{\ddagger}$) for the hydride transfer reaction with choline and 1,2-[$^2H_4$]-choline determined from the temperature dependence of the k_{cat}/K_m values are not significantly different from one another, with values of 18 ± 2 and 18 ± 5 kJ/mol, respectively (*26*). Finally, the isotope effect on the activation energy for the reaction (ΔE_a) is negligible, with a value of 0.4 ± 4 kJ/mol (*26*). These data taken together do not conform to a classical transition state for the reaction that is over the energetic barrier or to a semiclassical tunnelling correction with a transition state right below the energetic barrier for the reaction, but are consistent with the hydride ion tunnelling within a preorganized enzyme−substrate complex (*26*). Enzyme−substrate preorganization is maintained when the catalytic regime of the reaction is shifted from irreversible to reversible, which occurs when the enzyme turns over at subsaturating concentrations of oxygen due to the $k_4/k_5[O_2]$ ratio in Scheme 3 having a finite value (*30*). In this regard, the flux of reaction intermediates through the reverse of the hydride transfer step in choline oxidase changes with temperature, with the hydride transfer reaction becoming more reversible as the temperature increases from 10 to 45 °C (*30*). After correction for the kinetic complexity of the reaction arising from the $k_4/k_5[O_2]$ ratio having a finite value, analyses of the kinetic data according to both Arrhenius' and Eyring's formalisms demonstrate that the quantum mechanical nature of the hydride transfer reaction is maintained irrespective of whether the regime of catalysis shifts from irreversible to reversible (*30*). Evidence for quantum mechanical transfer of a hydride ion in flavin-dependent enzymes that oxidize alcohols is also available for a glucose oxidase form carrying extensive surface modifications when a glucose analogue is used as substrate (*4, 56*). With that enzyme, however, kinetic complexity does not allow one to draw conclusions on the wild-type form of the enzyme when the natural substrate glucose is used (*56*).

Preorganization of the Activated Enzyme−Substrate Complex. The environmental organization that is required to assemble the hydride ion donor and acceptor in the configuration that is necessary for the tunnelling of the hydride ion is attained through a conformational change of the activated enzyme−substrate complex that occurs prior to and independently from the hydride transfer reaction, as illustrated in Scheme 6. Evidence for such a conformational change, which is mechanistically uncoupled from the subsequent hydride transfer reaction, comes from the comparison of the thermodynamic parameters for the hydride transfer reaction catalyzed by wild-type choline oxidase under irreversible and reversible catalytic regimes (*30*). Indeed, the only difference seen in the thermodynamic parameters associated with the enzymatic reaction is a significant increase in the enthalpy of activation ($\Delta H^{\ddagger}$) for the reaction of hydride ion transfer carried out under a reversible catalytic regime with respect to the $\Delta H^{\ddagger}$ value for the reaction under an irreversible catalytic regime (~30 kJ/mol versus ~18 kJ/mol) determined in temperature-dependence studies (*30*). The conformational change of the activated enzyme−substrate complex is fast in the wild-type enzyme (*30*). In contrast, upon replacement of Glu312 with aspartate, which by virtue of anchoring the trimethylammonium moiety of the substrate necessarily moves the α-carbon of choline ~1 Å away from the flavin N(5) atom, results in an enzyme variant in which the conformational change of the enzyme−substrate complex becomes kinetically relevant, as suggested by solvent viscosity and substrate kinetic isotope effects on the k_{cat}/K_m value with choline (*20*). The conformational change of the activated enzyme−substrate complex that is required to preorganize the active site for the hydride transfer reaction appears to be associated with the enzyme-catalyzed removal of the hydroxyl proton of choline, which produces a negative charge on the alkoxide oxygen atom of the activated substrate that establishes an electrostatic interaction with the positively charged side chain of His466 (*18, 21*). This conclusion is supported by the observation that in enzyme variants where Val464 is replaced with threonine or alanine there is a significant effect of solvent viscosity on the rate constant for flavin reduction determined in anaerobic stopped-flow spectrophotometry with choline as substrate, which decreases by ~40% in the presence of viscosigens (*22*). Since a reaction of hydroxyl proton abstraction per se does not depend on solvent viscosity, the effect of solvent viscosity must necessarily arise from a solvent sensitive conformational change of the enzyme−choline Michaelis complex that equilibrates with the activated enzyme−choline alkoxide complex (Scheme 6). A conformational change of the enzyme−substrate complex that is coupled to the removal of the hydroxyl proton of the alcohol substrate similar to that seen with choline oxidase was also proposed for the

wild-type and a mutant variant of flavocytochrome b2 using solvent viscosity and kinetic isotope effects (*12, 44*).

The mechanistic conclusions drawn on the reaction of alcohol oxidation catalyzed by choline oxidase are fully supported by the structural information on the enzyme recently obtained through X-ray crystallography (*20*). In agreement with a preorganized activated enzyme−substrate complex, the active site cavity of choline oxidase has a volume of ~125 Å^3, which is slightly larger than the volume of 93 Å^3 estimated for choline. Moreover, the isoalloxazine ring of the flavin cofactor, which acts as a hydride ion acceptor during alcohol oxidation, is physically constrained by the covalent linkage of its C(8) methyl group with the side chain of His99, the proximity of Ile103 to the pyrimidine ring at the opposite end of the flavin ring system, and several contacts with the backbone atoms of His99, Asn100, Ser101, Cys102, and Ile103 (*20*). In a similar fashion, the choline alkoxide species that donates a hydride ion in the reaction is also physically constrained by electrostatic interactions of its positively charged amine headgroup with the side chain of Glu312, its negatively charged alkoxide oxygen atom at the opposite end with the positively charged side chain of His466 (*18, 21*), and a hydrogen bonding interaction of its oxygen atom with the side chain of His351 (*1*). It will be interesting to address future studies at the disruption of the activated enzyme−substrate preorganization by selectively replacing amino acid residues in the active site of the enzyme to investigate the impact of permitting independent movement of the hydride ion donor and acceptor on the mode and efficiency of hydride ion transfer in the reaction catalyzed by choline oxidase.

CONCLUSIONS

The mechanistic and structural studies carried out in the past five years have contributed to the delineation of the mechanism for the reaction of alcohol oxidation catalyzed by *A. globiformis* choline oxidase. The alcohol substrate is initially activated in the active site of the enzyme by a fast proton abstraction step on the hydroxyl group (Scheme 4) (*16, 23, 25, 27*). The resulting alkoxide species is stabilized in the activated enzyme−substrate complex via electrostatic and hydrogen bonding interactions with active site amino acid residues (Scheme 4) (*1, 18, 20, 21, 27*). Alcohol oxidation occurs through the transfer of a hydride ion from the α-carbon of the activated alkoxide species to the N(5) atom of the flavin cofactor in a quantum tunnelling fashion, through a reaction that is mechanistically and kinetically uncoupled to the proton abstraction step (Scheme 5) (*26, 27, 30*). Besides activating the enzyme−substrate complex, the formation of the choline alkoxide transient species that results from the uncoupling of OH and CH bonds cleavages is instrumental for the correct positioning of the alcohol substrate in the active site of the enzyme to allow for the hydride ion to be transferred quantum mechanically by exploiting the protein dynamical motions that enable a tunnelling distance between the substrate α-carbon and the flavin N(5) atom to be achieved (Scheme 6) (*26, 30*). A striking requirement for efficient hydride ion transfer is the high degree of preorganization of the activated enzyme−substrate complex, which is achieved through a conformational change occurring prior to and independently from the subsequent hydride transfer reaction (*20, 22, 30*). The importance of enzyme−substrate preorganization has therefore been emerging as an important factor controlling the oxidation reaction catalyzed by the enzyme.

Is there a General Strategy for Enzymatic Oxidation of Alcohols? To date, the enzymatic oxidation of alcohols has been shown to be catalyzed by flavin-dependent enzymes, zinc-dependent dehydrogenases, and pyrroloquinoline quinone-dependent dehydrogenases (*6, 10−12, 32, 33, 36, 37, 39−41, 48, 49, 57−69*). Other than choline oxidase, the best example for which an in-depth mechanistic characterization has been carried out is zinc-dependent alcohol dehydrogenase, which uses NAD(P)$^+$ as an electron acceptor. A consensus for the strategy employed by the enzyme to oxidize alcohols has been obtained through biochemical, mechanistic, computational, and structural studies (*70−75*). Briefly, an active site catalytic histidine is proposed to initiate catalysis with the activation of the alcohol substrate by removal of the hydroxyl proton. The resulting alkoxide is stabilized electrostatically in the active site by the positive charge provided by the enzyme-bound catalytic zinc. After completion of the proton transfer step, a hydride ion is transferred to the enzyme-bound pyridine dinucleotide through tunnelling within a highly preorganized activated enzyme−substrate complex. Moreover, a conformational change occurs upon binding of the substrate to the enzyme, which is required to correctly position the active site residues that participate in catalysis. Thus, the catalytic strategies for alcohol oxidation employed by the flavin-dependent choline oxidase and the zinc-dependent alcohol dehydrogenase appear to be similar to one another. In both cases, the substrate is activated before the subsequent oxidation step, the activated alkoxide intermediate and the transition state are electrostatically stabilized by positive charges, and the hydride ion tunnels quantum mechanically from the activated alkoxide to the isoalloxazine or pyridine dinucleotide acceptors. In this regard, recent studies on a variety of pyrroloquinoline quinone-dependent enzymes suggest the notion that these enzymes may also use a similar strategy for alcohol oxidation (*61, 76−79*), although further solution studies are required. Therefore, it is attractive to propose that a quantum mechanical tunnelling of a hydride ion from a chelated, activated alcohol to an organic cofactor perhaps is a common strategy utilized by enzymes that oxidize alcohols to the corresponding carbonyl compounds. While such a procurement would certainly be viewed as a simplistic approach to address the general problem of how enzymes catalyze alcohol oxidations, it will be interesting to establish experimentally whether the lessons that are still being learned on the mechanism of alcohol oxidation in the reaction catalyzed by choline oxidase can be extended to other flavin-dependent enzymes, as well as enzymes that utilize cofactors other than flavins, in the oxidation of alcohols.

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