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# Hydride Transfer Made Easy in the Oxidation of Alcohols Catalyzed by Choline Oxidase

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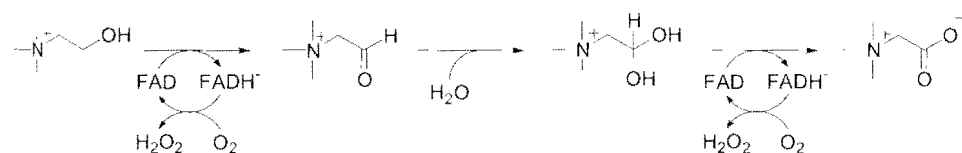
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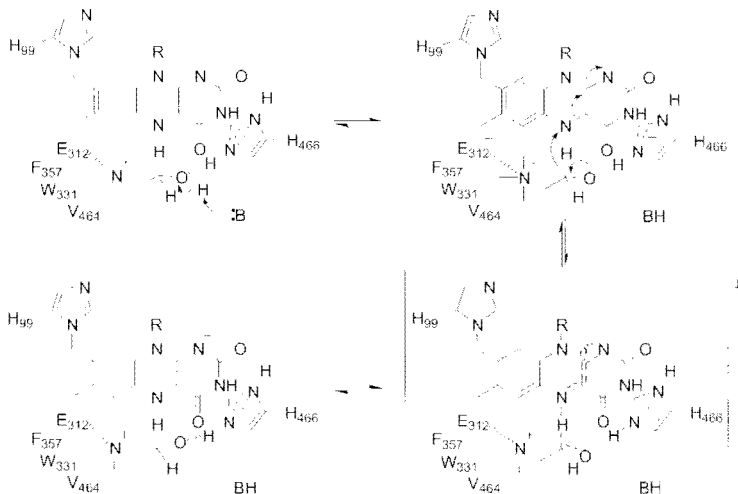
## Introduction

Choline oxidase (E.C. 1.1.3.17) 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 (Scheme 1) [1]. The *gem*-diol, hydrated species of the aldehyde intermediate of the reaction acts as substrate for aldehyde oxidation [2], suggesting that the enzyme may use similar strategies for the oxidation of the alcohol substrate and aldehyde intermediate.



**Scheme 1:** Two-step, four-electron oxidation of choline catalyzed by choline oxidase

The determination of the chemical mechanism for alcohol oxidation has emerged from biochemical [1, 3-6], mechanistic [2, 7-11], mutagenetic [12-14], and structural studies [14]. As illustrated in the mechanism of Scheme 2, the alcohol substrate is initially activated in the active site of the enzyme by removal of the hydroxyl proton [3, 4, 7]. The resulting alkoxide intermediate is then stabilized in the enzyme-substrate complex via electrostatic interactions with active site amino acid residues [12, 14]. Alcohol oxidation then occurs quantum mechanically via the transfer of the hydride ion from the activated substrate to the N(5) flavin locus [8, 9]. An essential requisite for this mechanism of alcohol oxidation is the high degree of preorganization of the activated enzyme-substrate complex, which is achieved through an internal equilibrium of the Michaelis complex occurring prior to, and independently from, the subsequent hydride transfer reaction [11, 14]. The experimental evidence that support the mechanism for alcohol oxidation shown in Scheme 2 is briefly summarized in the Results and Discussion section.



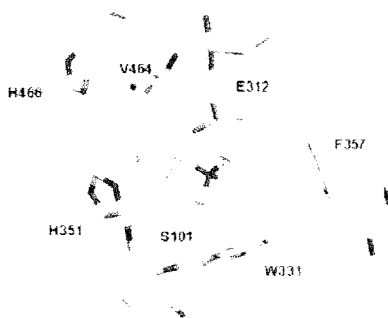
**Scheme 2:** Chemical mechanism for the oxidation of choline by choline oxidase

## Results and Discussion

### *Activation of the alcohol substrate*

The pH profiles for the steady state kinetic parameters have been determined for the wild type enzyme using choline and betaine aldehyde, and a number of substrate analogues, such as 1,2- $^{2}\text{H}_4$ -choline, *N,N*-dimethylethanolamine, *N*-methylethanolamine, and 3,3-dimethyl-butan-1-ol [2-4, 7, 8, 10]. In all cases, both the  $k_{\text{cat}}/K_m$  and  $k_{\text{cat}}$  pH profiles increase to limiting values with increasing pH, suggesting the requirement of a group with  $\text{p}K_a$  value of 7.5 to be unprotonated for catalysis. The value of 7.5 is a thermodynamic  $\text{p}K_a$ , as suggested by pH dependence studies of enzyme inhibition using glycine betaine [4]. A reasonable role for the active site group that needs to be unprotonated for catalysis is to act as a catalytic base for the abstraction

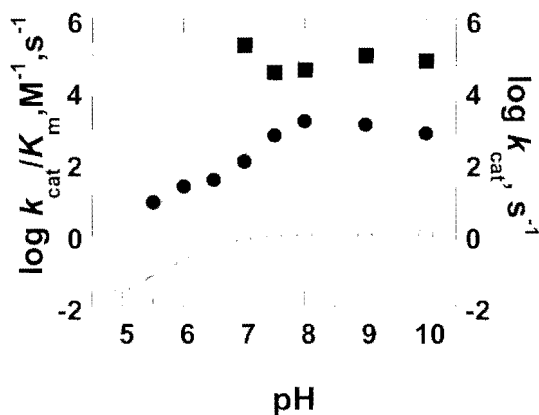
of the hydroxyl proton of the alcohol substrate (*top left panel of Scheme 2*). Two histidine residues are suitably located in the active site cavity of the enzyme with the potential to activate choline during turnover of the enzyme, namely His-351 and His-466 (Figure 1) [14]. Site-directed replacement of either residue with alanine results in mutant variants of the enzyme (H351A, Figure 2; H466A [12]) with significantly



**Figure 1:** The active site residues of choline oxidase (PDB 2jby). A hypothetical choline molecule has been docked in the center of the active site cavity.

lowered  $k_{\text{cat}}/K_m$  and  $k_{\text{cat}}$  values for choline at pH 10, i.e., the pH independent region of the pH profiles.

Interestingly, both single mutants show pH profiles for the  $k_{\text{cat}}/K_m$  and  $k_{\text{cat}}$  values that are still consistent with the presence of an unprotonated group that is required for catalysis, with thermodynamic  $pK_a$  values of 8.0 and 9.0 for H351A (data not shown) and H466A [12], respectively. These data suggest that neither of the two histidine residues is solely responsible for the activation of the choline substrate. Instead, they may either act together in the abstraction of the hydroxyl proton of the alcohol substrate, or substitute for each other when one is replaced by alanine. Studies on double mutant variants in which both histidine residues have been replaced with other amino acids are currently in progress to address this issue.



**Figure 2:** pH profiles for the  $k_{\text{cat}}/K_m$  (●),  $k_{\text{cat}}$  (○), and  $k_{\text{cat}}/K_{\text{oxygen}}$  (■) values with choline as substrate for the H351A variant of choline oxidase.

#### *Stabilization of the alkoxide intermediate*

Substrate and solvent kinetic isotope effects on the  $k_{\text{cat}}/K_m$  and  $k_{\text{red}}$  values with choline at pH 10 have demonstrated that cleavage of the OH bond of choline occurs prior to, and completely decoupled from, the subsequent hydride transfer reaction that results in the cleavage of the CH bond (Scheme 2) [9]. Mechanistic data for mutant forms of choline oxidase in which Glu-312 [14] and His-466 [12, 13] have been replaced by other amino acids 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 Glu-312 interacts electrostatically with the positively charged trimethylammonium moiety of the organic substrate and, by extension, with the intermediates and the product of the enzymatic reaction. Indeed, replacement of Glu-312 with glutamine results in a 500-fold increase in the  $K_d$  value for choline, as determined using rapid kinetics techniques [14]. This corresponds to an energetic contribution of  $\sim 15$  kJ/mol, in good agreement with the estimate of  $\sim 13$  kJ/mol that was independently determined from mechanistic studies with the choline analogue lacking the positive charge, i.e., 3,3-dimethyl-butan-1-ol [10]. Mechanistic, spectroscopic, and biochemical studies on the enzyme variant in which His-466 is replaced with alanine strongly support the notion that His-466 is protonated during the reductive half-reaction in which choline is oxidized to betaine aldehyde (Scheme 2) [12]. 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 [12].

#### *Quantum mechanical hydride transfer from the activated substrate to the flavin*

The effect of oxygen and temperature on the kinetic isotope effects on the steady state kinetic parameters  $k_{\text{cat}}/K_m$  and  $k_{\text{cat}}$  with 1,2- $^2\text{H}_4$ -choline as substrate as a function of pH have demonstrated that oxygen availability modulates whether the reduced enzyme-betaine aldehyde complex partitions forward to catalysis rather than reverting to the oxidized enzyme-choline alkoxide species [8]. At saturating concentrations of oxygen, i.e., under an irreversible catalytic regime, the  $D(k_{\text{cat}}/K_m)$  value is  $10.6 \pm 0.6$  and temperature independent [8]. The corresponding isotope effect on the Eyring preexponential factors ( $A_H'/A_D'$ ) is significantly larger than unity ( $14 \pm 3$ ) [8]. Furthermore, the enthalpies of activation ( $\Delta H^\ddagger$ ) for the hydride transfer reaction with choline and 1,2- $^2\text{H}_4$ -choline are not significantly different from each other, with values of  $18 \pm 2$  and  $18 \pm 5$  kJ/mol, respectively [8]. All taken together, these data are consistent with a mechanism of hydride transfer in which the hydride tunnels quantum mechanically from the  $\alpha$ -carbon of the alkoxide species to the N(5) atom of the enzyme-bound flavin within a highly preorganized activated enzyme-substrate complex (Scheme 2) [8]. The effects of pH and temperature on the substrate kinetic isotope effects with 1,2- $^2\text{H}_4$ -choline have been studied also at the subsaturating concentration of oxygen of 0.2 mM, to gain insights into the mechanism of hydride transfer under a reversible catalytic regime [11]. The flux of reaction intermediates through the reverse of the hydride transfer step, i.e., the so-called reverse commitment to catalysis, changes with temperature, with the hydride transfer reaction becoming more reversible as the temperature increases from 10 °C to 45 °C [11]. After correction for the kinetic complexity of the reaction due to the reverse commitment to catalysis, 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 [11]. However, the comparison of the thermodynamic parameters for the hydride transfer reaction under reversible and irreversible catalytic regimes unveils an enthalpically unfavourable internal equilibrium of the enzyme-substrate complex that occurs prior to the hydride transfer reaction [11]. Such an internal equilibrium is kinetically undetectable in the wild type enzyme, and is likely required to preorganize the activated enzyme-substrate complex for the efficient quantum mechanical transfer of the hydride from the substrate  $\alpha$ -carbon to the N(5) atom of the enzyme-bound flavin. Interestingly, replacement of Glu-312 with aspartate results in an enzyme variant in which the conformational change of the enzyme-substrate complex becomes kinetically relevant, as suggested by the effects of solvent viscosity on the  $k_{\text{cat}}/K_m$  value with choline and substrate kinetic isotope effects [14].

### *Preorganization of the activated enzyme-substrate complex*

The results of the crystallographic and mechanistic studies on choline oxidase reported thus far are consistent with the active site of the enzyme being well-suited to maximize several aspects of the oxidation reaction. Choline is bound productively through its trimethylammonium group so that alcohol activation can be efficiently achieved by the action of the catalytic base. Once alcohol activation is attained, the resulting negatively charged alkoxide species is locked in position through electrostatic interaction with a positive charge provided by the side chain of His-466. Flavin movement with respect to the organic substrate is also significantly restricted due to the covalent linkage of the FAD C8M atom with the N $\epsilon$ 2 atom of His-99 [14]. Thus, very little independent movement of the hydride donor and acceptor is allowed in the activated enzyme-substrate complex, thereby rendering the quantum mechanical transfer of the hydride ion from the alkoxide  $\alpha$ -carbon to the flavin N(5) atom the most favoured outcome for the reaction. Currently, site-directed replacements of amino acids that do not participate directly in the oxidation of choline to betaine aldehyde in the active site of choline oxidase are under mechanistic investigation in order to establish how the hydride transfer reaction is affected by the disruption of preorganization in the enzyme-substrate activated complex.

### *Conclusions*

The mechanistic and structural studies reported thus far have contributed to the delineation of the mechanism for alcohol oxidation in the reaction catalyzed by choline oxidase (Scheme 2). The importance of enzyme-substrate preorganization in the active site of the enzyme has been emerging as an important factor for the efficient oxidation reaction catalyzed by the enzyme. Future mechanistic studies will be aimed at the disruption of such enzyme-substrate preorganization in order to establish the impact on the hydride transfer reaction catalyzed by the enzyme. Finally, choline oxidase has been grouped in a superfamily of flavin-dependent enzymes that oxidize alcohols to the corresponding carbonyl compounds, the Glucose-Methanol-Choline Oxidoreductases. The crystal structures of several members of the superfamily, including glucose oxidase, cholesterol oxidase, pyranose 2-oxidase, cellobiose dehydrogenase, and choline oxidase itself, are currently available, showing that the active sites of these enzymes contain similar, if not identical, amino acid residues. Therefore, it will be interesting to establish whether the lessons we are continuing to learn regarding the mechanism of alcohol oxidation in the reaction catalyzed by choline oxidase can be extended to the rest of the family members.

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