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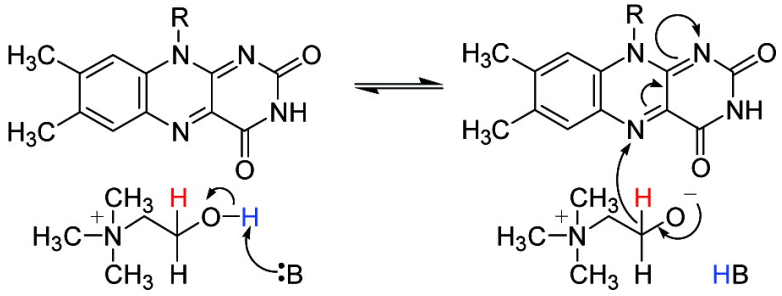

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On the Catalytic Mechanism of Choline Oxidase

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Abstract: Choline oxidase catalyzes the four-electron oxidation of choline to glycine betaine, with betaine aldehyde as an intermediate. In this study, primary deuterium and solvent kinetic isotope effects have been used to elucidate the mechanism for substrate oxidation by choline oxidase using both steady-state kinetics and rapid kinetics techniques. The $^{D}(k_{cat}/K_{m})$ value with 1,2-[$^{2}H_{4}$]-choline at saturating oxygen concentration was independent of pH in the range between 6.5 and 10, with a value of ~10.6, indicating that CH bond cleavage is not masked by other titratable kinetic steps belonging to the reductive half-reaction. In agreement with this conclusion, a $^{D}k_{red}$ value of ~8.9 was determined at pH 10 for the anaerobic reduction of the flavin by choline, irrespective of whether aqueous or deuterated solvent was used. At pH 10, both the $^{D_2O}(k_{cat}/K_{m})$ and the $^{D_2O}k_{red}$ values were not different from unity with choline or 1,2-[$^{2}H_{4}$]-choline, while the $^{D}k_{cat}$ and $^{D_2O}k_{cat}$ values were 7.3 and 1.1, respectively. The k_{cat} and k_{red} values were 133 s^{-1} and 135 s^{-1} with betaine aldehyde and 60 s^{-1} and 93 s^{-1} with choline. These data are consistent with a chemical mechanism in which the choline hydroxyl proton is not in flight in the transition state for CH bond cleavage and with chemical steps of flavin reduction by choline and betaine aldehyde being rate limiting for the overall turnover of the enzyme.

The oxidation of alcohols to carbonyl compounds is a very important chemical reaction that is central to a number of biochemical pathways, such as the polyol pathway of glucose metabolism,[1] butane oxidation pathway,[2] and D-xylose catabolism.[3] To date, enzymes with the ability to oxidize alcohols have been shown to utilize zinc,[1,3−6] pyrroloquinoline quinone (PQQ),[2,7−13] or flavins[14−20] as cofactors for the reaction. A number of flavin-dependent enzymes that oxidize unpolarized alcohols and share a highly conserved catalytic site have been grouped in the GMC oxidoreductase enzyme superfamily,[17,21−24] which includes choline oxidase, choline dehydrogenase, glucose oxidase, cholesterol oxidase, methanol oxidase, and cellobiose dehydrogenase. While general consensus for the chemical mechanism of reaction has been obtained for the zinc- and PQQ-dependent alcohol dehydrogenases,[7,12,25,26] several possible mechanisms have been suggested for the GMC enzymes.[18,22,27] Structural studies on glucose oxidase,[14−16] cholesterol oxidase,[18−20] and the flavin domain of cellobiose dehydrogenase[17,27] support the notion that catalysis is carried out by removal of the hydroxyl proton by an active site base and concomitant transfer of a hydride from the substrate α-carbon to the flavin cofactor (Scheme 1, path a). However, mechanistic studies using kinetic isotope effects to probe the relative timing of OH and CH bond

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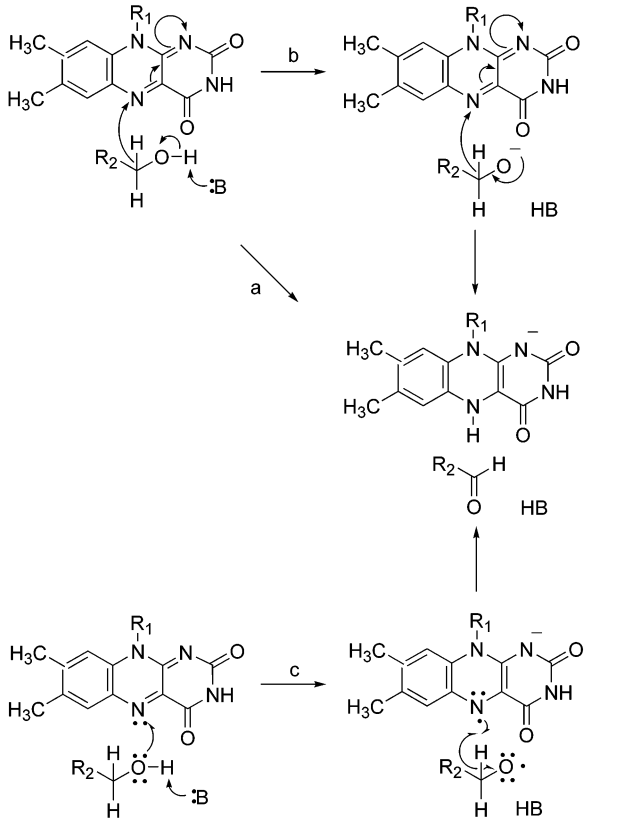
Scheme 1. Proposed Mechanisms for Alcohol Oxidation by GMC Enzymes[a]



[a] (a) Hydride transfer mechanism, (b) asynchronous hydride transfer mechanism, (c) oxygen radical mechanism.

cleavages have been greatly hindered in glucose oxidase[28] and cholesterol oxidase[29,30] due to the occurrence of steps that are slower than those that directly involve the reductive or oxidative processes. In an elegant study on methanol oxidase combining the use of isotope effects and substrate analogues, an asynchronous hydride transfer mechanism in which OH bond cleavage occurs to a great extent before CH bond cleavage was proposed (Scheme 1, path b).[31] In an alternative mechanism that has been suggested in structural studies on cholesterol oxidase and cellobiose dehydrogenase,[18,27] a single electron is transferred to the flavin concomitantly with abstraction of the hydroxyl proton, followed by transfer of the α-hydrogen to the flavin (Scheme 1, path c). However, biochemical studies have failed thus far to provide evidence for the formation of the two radical species involved in such a mechanism. A carbanion mechanism, which was originally proposed for the oxidation of polarized alcohols catalyzed by flavocytochrome b_2,[32,33] is the least likely for the oxidation of unpolarized alcohols due to energetic considerations related to the stabilization of the incipient negative charge.[34,35] In this mechanism, catalysis would be initiated by abstraction of the substrate α-proton by an active site base to form a carbanion, followed by formation of a covalent N(5)-flavin adduct that would subsequently decay yielding the aldehyde product and reduced flavin.

Our group recently cloned and expressed the gene coding for choline oxidase (E.C. 1.1.3.17) from *Arthrobacter globiformis* strain ATCC 8010[23] and showed that the resulting enzyme maintains biochemical and kinetic properties similar to those of the native choline oxidase.[23,36] The enzyme contains covalently bound FAD in a 8α-N(1)-histidyl linkage[37] and catalyzes the oxidation of choline to glycine betaine via betaine aldehyde as intermediate.[23,38,39] The steady-state kinetic mechanism of choline oxidase was recently reported and is consistent with two sequential flavin-linked oxidations of choline and the ensuing betaine aldehyde, each followed by the oxidation of the reduced flavin by molecular oxygen in the betaine aldehyde− and glycine betaine−enzyme complexes (Scheme 2).[36,39] A catalytic base with a pK_a of 7.5 participates in the oxidation of choline, but not in the reduction of oxygen.[36,40] Preliminary investigations of the enzyme using deuterated choline at fixed oxygen concentration showed large kinetic isotope effects,[22] suggesting that choline oxidase might be a good candidate for the elucidation of the mechanism of bond cleavage in the GMC enzymes. In the study described herein, primary deuterium and solvent kinetic isotope effects, in combination with steady-state and rapid kinetics approaches, have been used to probe the relative timing of OH and CH bond cleavage in choline oxidase. The results of these studies provide insights into the mechanism of oxidation of unpolarized alcohols catalyzed by choline oxidase and, by extension, of the members of the GMC oxidoreductase enzyme superfamily.

Experimental Procedures

Materials. Choline chloride was from ICN. Betaine aldehyde chloride was from Sigma. 1,2-[$^2H_4$]-Choline bromide (98%) and sodium deuterioxide (99%) were from Isotec Inc. (Miamisburg, OH). Deuterium chloride (99.5%) and deuterium oxide (99.9%) were from Cambridge Isotope Co. (Andover, MA). All other reagents were of the highest purity commercially available. Recombinant choline oxidase from *A. globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described previously.[23] Choline oxidase as purified, containing a mixture of catalytically competent oxidized flavin and noncatalytically competent anionic semiquinone,[36] was used for steady-state kinetics, whereas choline oxidase with 100% oxidized flavin was used for rapid kinetics. Fully oxidized enzyme was prepared as described previously.[40] All kinetic parameters determined were expressed per active site oxidized flavin content as determined previously.[36]

Kinetic Assays. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode thermostated at 25 °C. The determination of the steady-state kinetic parameters was carried at varying concentrations of both choline (or betaine aldehyde), in the range from 0.02 to 15 mM, and oxygen, in the range from 0.04 to 1.1 mM. The reaction mixture was first equilibrated at the desired concentration of oxygen by bubbling the appropriate O_2/N_2 gas mixture for at least 10 min. The reactions were

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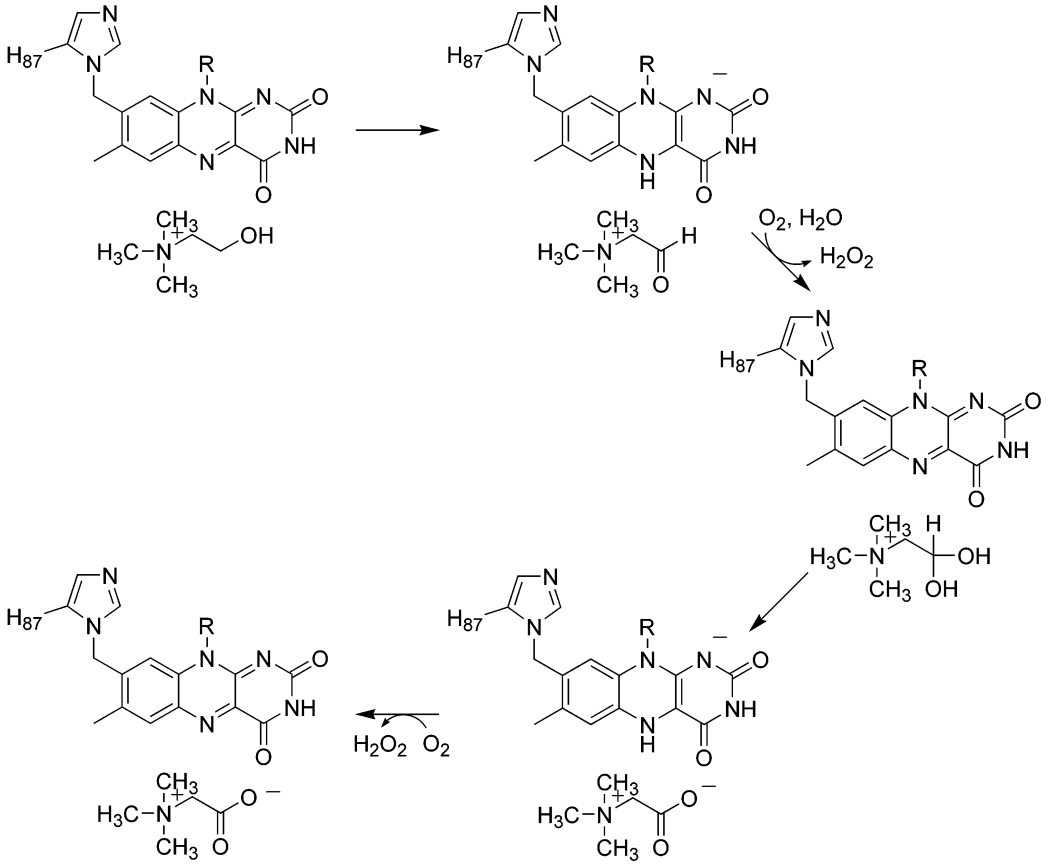
Scheme 2. Oxidation of Choline by Choline Oxidase



then started by adding 5 μL of choline oxidase at a final concentration of ~0.1 μM into a reaction mixture with a final volume of 1 mL. Enzyme assays were conducted in 50 mM sodium pyrophosphate, except for pH 7 and 7.5 where potassium phosphate was used.

The deuterium isotope effects on steady-state kinetic parameters were determined using 1,2-[$^2H_4$]-choline as substrate at varying oxygen concentrations. For the determination of solvent isotope effects, buffers were prepared using 99.9% deuterium oxide by adjusting the pD value with DCl or NaOD. The pD values were determined by adding 0.4 to the pH electrode readings.[41] For all steady-state kinetic isotope effects, activity assays were carried out by alternating substrate or solvent isoptomers. The effects of solvent viscosity on steady-state kinetic parameters were measured in 50 mM sodium pyrophosphate, pH 10, at 25 °C, using glycerol as viscogen. The values for the relative viscosities at 25 °C were calculated according to the values at 20 °C from Lide.[42]

Rapid kinetics was carried out on a Hi-Tech SF-61 stopped-flow spectrophotometer thermostated at 25 °C. The rate of flavin reduction was measured by monitoring the decrease in absorbance at 452 nm that results from the decreasing of oxidized flavin species upon mixing the enzyme with the substrate. For this experiment, choline oxidase was first lyophilized and then dissolved in 100 mM sodium pyrophosphate in either aqueous solvent or D_2O, the pL value of which was adjusted to 10. The resulting enzyme solution was loaded into a tonometer and subjected to a 23-cycle degassing procedure by alternately applying vacuum and flushing with oxygen-free argon (pretreated with an oxygen scrubbing cartridge, Agilent, Palo Alto, CA). Subsequently, the degassed enzyme solution was mounted onto the stopped-flow instrument, which had been subjected to an overnight treatment with an oxygen scrubbing system composed of 100 mM glucose and 30 units mL^{-1} glucose oxidase. The substrate, choline or 1,2-[$^2H_4$]-choline (~2 mL), was dissolved in H_2O or D_2O and then degassed by flushing with oxygen-free argon for at least 15 min before mounting onto the stopped-flow. The enzyme was mixed anaerobically with an equal volume of substrate, yielding a reaction mixture containing ~15 μM choline oxidase and 0.05 to 10 mM choline or 1,2-[$^2H_4$]-choline, in either aqueous or deuterated 50 mM sodium pyrophosphate, pL 10. The effects of isotopically labeled substrate or solvent on the rapid kinetics were determined by alternating substrate or solvent isoptomers. For each concentration of the substrate, the activity was assayed in triplicate and the average value was considered. Typically, measurements differed by ≤5%.

With betaine aldehyde as substrate, steady-state kinetic parameters were measured at varying concentrations of betaine aldehyde (between 0.05 and 5 mM) and oxygen (in a range between 0.1 and 0.5 mM). The reductive half reaction was monitored anaerobically with choline oxidase at a final concentration of ~15 μM and betaine aldehyde in a range between 0.05 and 5 mM using a stopped-flow spectrophotometer, at pH 10 and 25 °C.

Data Analysis. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA) and Enzfitter software (Biosoft, Cambridge, UK). The steady-state kinetic parameters at varying concentrations of both choline and oxygen were determined by fitting the initial rate data to eqs 1 and 2, which describe steady-state kinetic mechanisms with intersecting and parallel lines in double reciprocal plots, respectively.

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

$$\frac{v}{e} = \frac{k_{cat}AB}{K_aB + K_bA + AB} \quad (2)$$

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Data with betaine aldehyde were fit into eq 3, which describes a tenary complex mechanism with negligible K_a value.

$$\frac{v}{e} = \frac{k_{cat}AB}{K_bA + AB + K_{ia}K_b} \quad (3)$$

In these equations, e represents the concentration of enzyme, k_{cat} is the turnover number of the enzyme at infinite substrates concentrations, K_a and K_b represent the Michaelis constants for the organic substrate (A) and oxygen (B), respectively. For the kinetic isotope effects with choline as substrate, data obtained were divided into two sets, one with unlabeled substrate or solvent and one with isotopically labeled substrate or solvent. The steady-state kinetic parameters of the two sets were determined independently with eq 1 or 2, and the kinetic isotope effects were determined by taking the ratios of the steady-state kinetic parameters of interest.[43] Stopped-flow traces were fit into eq 4 that describes a case of single-exponential decay, in which k_{obs} represents the first-order rate constant for the reductive half reaction, t is time, A_t is the value of absorbance at 452 nm, A is the amplitude of the total change, and A_∞ is the absorbance at infinite time.

$$A_t = A\exp(-k_{obs}t) + A_\infty \quad (4)$$

The pre-steady-state kinetic parameters were determined by using eqs 5 and 6, where k_{obs} is the observed rate for the reduction of enzyme bound flavin, k_{red} is the limiting rate of flavin reduction at saturated substrate concentration, K_d is the dissociation constant, and k_{rev} is the rate of the reverse step in catalysis.

$$k_{obs} = \frac{k_{red}A}{K_d + A} + k_{rev} \quad (5)$$

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

The substrate and solvent deuterium isotope effects on rapid kinetic parameters were determined by using eq 7, which describes a sequential mechanism with separate isotope effects on both k_{red} and k_{red}/K_d.

$$\frac{v}{e} = \frac{k_{red}A}{K_d(1 + F_iE_{k_{red}/K_d}) + A(1 + E_{k_{red}})} \quad (7)$$

F_i is the atom fraction of deuterium label in the substrate. E_{k_{red}/K_d} and $E_{k_{red}}$ are the isotope effects minus one on k_{red}/K_d and k_{red}, respectively. The viscosity effects on k_{cat}/K_m values for the organic substrate and oxygen and the k_{cat} value were fit to eq 8, where $(k)_o$ and $(k)_\eta$ are the kinetic parameters of interest in the absence and presence of viscogen, respectively, S is the degree of viscosity dependence, and η_{rel} is the relative viscosity.

$$\frac{(k)_o}{(k)_\eta} = S(\eta_{rel} - 1) + 1 \quad (8)$$

Results

Previous studies on choline oxidase showed that when the concentrations of both choline and oxygen are varied, the steady-state kinetic data fit best by using eq 1, consistent with the formation of a ternary complex that involves the enzyme, the organic product, and molecular oxygen.[36,39] Consequently, kinetic isotope effects using the steady-state approach were determined at varying concentrations of both the organic substrate and oxygen. Due to K_m values for oxygen with 1,2-[$^2H_4$]-choline as substrate being in the low μM range, double reciprocal plots of the initial rate of reaction as a function of 1,2-[$^2H_4$]-choline concentration yielded parallel lines (data not shown). As a result, unequivocal determinations of both K_{ia} and K_m values for oxygen could not be obtained by using eq 1. In contrast, significant improvements in the quality of the fits, as reflected in the R^2 value, were obtained when the kinetic data with deuterated choline were fit to eq 2.

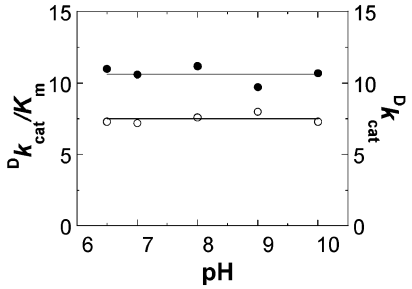

Figure 1. pH-Dependence of deuterium isotope effects on k_{cat}/K_m values (●) and k_{cat} values (○). Activity assays of choline oxidase were performed in 50 mM buffer with choline and 1,2-[$^2H_4$]-choline as substrate, at 25 °C. Data were fit by $y = 10.6$ and $y = 7.5$, for k_{cat}/K_m and k_{cat} values, respectively.

Table 1. Steady State and Pre-Steady-State Kinetic Parameters for Choline Oxidase[a]

parameter	choline	betaine aldehyde
k_{cat}, s^{-1}	60 ± 1	133 ± 4
k_{cat}/K_m, $M^{-1}\ s^{-1}$	237 000 ± 9000	nd[b]
k_{cat}/K_{O_2}, $M^{-1}\ s^{-1}$	86 400 ± 3600	53 400 ± 1600
K_m, mM	0.25 ± 0.01	nd
K_{O_2}, mM	0.69 ± 0.03	2.54 ± 0.01
K_{ia}, mM	0.14 ± 0.01	2.14 ± 0.08
k_{red}, s^{-1}	93 ± 1	135 ± 4
K_d, mM	0.29 ± 0.01	0.45 ± 0.03

[a] Conditions: 50 mM sodium pyrophosphate, pH 10, at 25 °C. Steady-state kinetic parameters with choline and betaine aldehyde were fit to eqs 1 and 3, respectively. Pre-steady-state kinetic parameters were fit to eq 6. In all cases, $R^2 \geq 0.998$. [b] nd, not determined.

As shown in Figure 1, the $^D(k_{cat}/K_m)$ and $^Dk_{cat}$ values measured at saturating concentrations of oxygen were pH-independent, with mean values of 10.6 ± 0.6 and 7.5 ± 0.3, respectively, suggesting that cleavage of the choline CH bond is not masked by other kinetic steps belonging to the reductive half-reaction or the overall turnover and a lack of any forward or reverse commitment.[44] Tables 1 and 2 illustrate the kinetic parameters and the kinetic isotope effects at saturating oxygen determined at pH 10, respectively.

The effect of deuterated solvent was determined to probe the timing of kinetic steps involving solvent exchangeable protons, such as the cleavage of the OH bond in choline. Since pK_a values change significantly with the isotopic composition of the solvent resulting in the shifts of the pH-profiles in deuterated aqueous environments,[41,45] the $^{D_2O}(k_{cat}/K_m)$ and $^{D_2O}k_{cat}$ values were determined above pL 9 to avoid artifactual contributions arising from pH effects. Indeed, previous pH-dependence studies on choline oxidase showed that both k_{cat}/K_m and k_{cat} become pH-independent at high pH.[22,36,40] At both pL 9 and 10, the ^{D_2O}-

(43) The equation $(v/e) = (k_{cat}AB/K_a[1 + F_i(E_{k_{cat}/K_m})]B + K_bA[1 + F_i(E_{k_{cat}})] + A[1 + F_i(E_{k_{cat}})]B + K_{ia}K_b)$ cannot be used for the determination of deuterium isotope effects under steady-state conditions, since the best fits of the kinetic data with choline and 1,2-[$^2H_4$]-choline were obtained by using eqs 1 and 2, respectively.

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Table 2. Substrate and Solvent Kinetic Isotope Effects with Choline as Substrate[a]

parameter	value
$^{D}k_{red}$	8.9 ± 0.2
$^{D}(k_{red})_{D_2O}$	8.7 ± 0.2
$^{D,D_2O}k_{red}$	8.4 ± 0.2
$^{D}k_{cat}$	7.3 ± 1.0
$^{D,D_2O}k_{cat}$	7.3 ± 0.2
$^{D}(k_{cat}/K_m)$	10.7 ± 2.6
$^{D,D_2O}(k_{cat}/K_m)$	10.4 ± 1.8
$^{D_2O}k_{red}$	0.99 ± 0.02
$^{D_2O}(k_{red})_D$	0.94 ± 0.03
$^{D_2O}k_{cat}$	1.1 ± 0.1
$^{D_2O}(k_{cat}/K_m)$	1.1 ± 0.2

[a] Conditions: 50 mM sodium pyrophosphate, pH 10, at 25 °C. The standard deviation for each k_{cat}/K_m and k_{cat} values was calculated by multiplying the value determined for the experimental parameter by the square root of the sum of the squares of the percent errors contributed by each experimental parameter; the standard deviation for each k_{red} value was determined by using eq 6.

(k_{cat}/K_m) and $^{D_2O}k_{cat}$ values determined were not significantly different from unity. Table 2 summarizes the solvent kinetic isotope effect values determined at pL 10. The relative timing of CH and OH bind cleavage was determined by measuring multiple kinetic isotope effects at varying concentrations of oxygen at pL 10. The ratio of the kinetic parameters with choline in H_2O to those with deuterated choline in D_2O yielded ^{D,D_2O}-(k_{cat}/K_m) and $^{D,D_2O}k_{cat}$ values that were not significantly different from the $^{D}(k_{cat}/K_m)$ and $^{D}k_{cat}$ values (Table 2).

A possible complication in the analysis of kinetic isotope effects determined from steady-state methods might arise from solvent sensitive kinetic steps that do not involve hydroxyl proton abstraction directly, changing the contribution of OH bond cleavage to the observed solvent isotope effect.[46] Since the viscosity of D_2O at 25 °C is ~23% greater than that of H_2O,[45] the effect of increasing solvent viscosity on k_{cat}/K_m and k_{cat} was measured at varying oxygen concentrations at pH 10 to probe for the presence of solvent sensitive steps in the reductive half-reaction and overall turnover. As shown in Figure 2, the normalized k_{cat}/K_m value at increasing concentrations of glycerol as viscogen was independent of solvent viscosity, ruling out internal equilibria of the enzyme−substrate complex occurring before the catalytic step of the kind observed with flavocytochrome b_2.[47] In a similar fashion, no significant effect of the solvent viscosity was observed on the k_{cat} value (Figure 2), suggesting that the overall turnover number is not limited by product release.

Previous kinetic studies on choline oxidase indicated that the k_{cat}/K_m value for oxygen is pH-independent between pH 6 and 10, irrespective of whether choline or betaine aldehyde is used as the organic substrate.[36] In this study, no significant changes were observed in the k_{cat}/K_m values for oxygen upon substituting choline with 1,2-[$^2H_4$]-choline, irrespective of whether H_2O, D_2O, or solvents with increased viscosity were used, indicating that the reactivity of the enzyme-bound reduced flavin toward oxygen is not affected by isotopic substitutions in the substrate or solvent or by solvent viscosity. A representative value for the k_{cat}/K_m value for oxygen at pH 10 with choline in aqueous solvent is shown in Table 1.

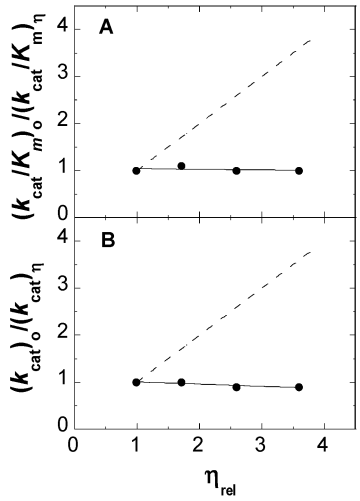


Figure 2. Effect of solvent viscosity on steady-state kinetic parameters. Top panel shows the effect on the k_{cat}/K_m value. Bottom panel shows the effect on the k_{cat} value. Solid line represents the experimental data. The dashed line with a slope of one describes a case in which the reaction is diffusion-controlled. The values for the relative viscosities of buffer at 25 °C were calculated according to the values at 20 °C from Lide.[42] Activity assays of choline oxidase were performed by varying both concentrations of oxygen and choline in 50 mM sodium pyrophosphate, at 25 °C. The solid lines represent fits of the data to eq 8.

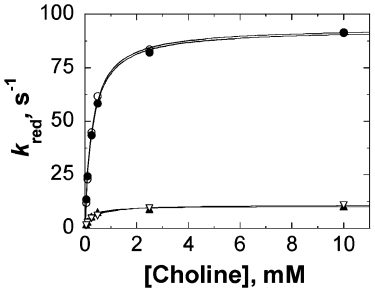


Figure 3. Rate of flavin reduction as a function of choline concentration. Experiments were carried out with choline in H_2O (●), 1,2-[$^2H_4$]-choline in H_2O (▲), choline in D_2O (○), and 1,2-[$^2H_4$]-choline in D_2O (▽). Assays were performed in 50 mM sodium pyrophosphate, pH 10, at 25 °C. Data were fit into eq 6.

As an independent approach by which the chemical step in the reductive half-reaction can be directly probed, kinetic isotope effects were determined for the rate of flavin reduction by choline using rapid reaction techniques at pH 10. Upon mixing choline oxidase with choline in the absence of oxygen, complete reduction of the flavin without formation of detectable transient species was observed following a first-order process, allowing for the determination of k_{red} values. When the observed rates of flavin reduction as a function of the concentration of organic substrate were fit by eq 5, a negligible rate constant for the reverse of the flavin reduction step, k_{rev}, was determined (data not shown). Accordingly, the kinetic data were fit equally well by eq 6, in which the rate of hydride transfer from the substrate α-carbon to the flavin is assumed to be significantly larger than the rate for the reverse reaction (Figure 3). As shown in Table 2, the $^{D}k_{red}$ and $^{D_2O}k_{red}$ values were comparable to the effects seen on their steady-state k_{cat}/K_m counterparts, consistent with flavin reduction being primarily rate limiting. The $^{D}k_{red}$ value was the same irrespective of whether H_2O or D_2O was the solvent; analogously, the $^{D_2O}k_{red}$ value did not change significantly upon substituting choline with deuterated choline. The

(46) Karsten, W. E.; Lai, C. J.; Cook, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 5914−5918.
(47) Sobrado, P.; Daubner, S. C.; Fitzpatrick, P. F. *Biochemistry* **2001**, *40*, 994−1001.

ratio of the k_{red} value with choline in H_2O to that with 1,2-[$^2H_4$]-choline in D_2O was not significantly different from the $^Dk_{red}$ value. All taken together, these data are consistent with hydroxyl proton abstraction and hydride transfer occurring in two different kinetic steps.

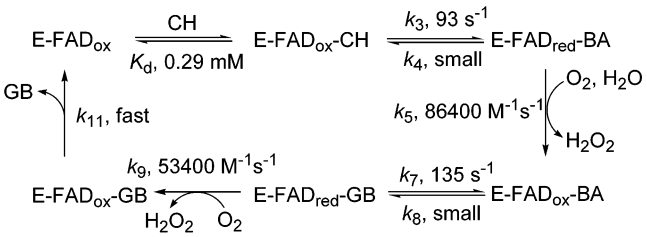
During turnover of choline oxidase, betaine aldehyde is further oxidized to glycine betaine before dissociating from the active site of the enzyme, as shown by earlier titrations of the betaine aldehyde that is released in solution when the enzyme turns over with choline.[39] Accordingly, the k_{cat} value with choline as substrate includes all the kinetic steps occurring after formation of the enzyme−choline complex until glycine betaine release.[36,39,48] Therefore, a detailed understanding of the kinetic steps that limit the overall turnover of the enzyme with choline requires knowledge of the rates of the kinetic steps occurring after formation of betaine aldehyde. Consequently, both the steady-state kinetic parameters and the rate of reduction of the flavin by betaine aldehyde were measured at pH 10. As for the case with choline, the flavin was reduced anaerobically by betaine aldehyde to the hydroquinone form in a single exponential process of the first-order, consistent with lack of any detectable transient species. The kinetic data were fit equally well by using eqs 5 and 6, suggesting that the rate of flavin reduction is significantly larger than the rate for the reverse reaction (data not shown). As expected from earlier studies on choline oxidase, the steady-state kinetic data with betaine aldehyde were fit best by eq 3, suggesting that the K_m value for betaine aldehyde is significantly smaller than the K_{ia} value of 2.1 mM, with a likely value in the low μM range.[36,39] As shown in Table 1, at pH 10 the k_{red} value was comparable in size to the k_{cat} value and was slightly larger than the rate of flavin reduction with choline.

Discussion

Kinetic isotope effects with isotopically substituted substrate and solvent have been used in the present study to obtain insights into the mechanism of alcohol oxidation in the reaction catalyzed by choline oxidase. This reaction involves the removal of both the hydroxyl proton and the hydrogen bound to the α-carbon of the alcohol substrate, i.e., choline, in either a concerted or stepwise fashion, depending upon the relative timing of CH and OH bond cleavage (Scheme 1). Primary deuterium kinetic isotope effects provide a direct probe of the status of the CH bond, whereas the effect of deuterated solvent can be used to provide information on the status of the OH bond.

The overall turnover with choline is limited by both chemical steps in which hydride equivalents are transferred from the choline substrate and the aldehyde intermediate to the enzyme-bound flavin. Both chemical steps of flavin reduction are significantly faster in the forward direction relative to the reverse direction, as suggested by the stopped-flow analyses of the reductive half-reactions with choline and betaine aldehyde. Moreover, release of glycine betaine from the enzyme is significantly faster than kinetic steps that limit the overall turnover, as suggested by the lack of viscosity effects on the k_{cat} value. Accordingly, the k_{cat} value with choline is given by

Scheme 3. Minimal Steady-State Kinetic Mechanism by Choline Oxidase at pH 10[a]



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

eq 9, where k_3 and k_7 represent the rate constants for flavin reduction by choline and betaine aldehyde, respectively (Scheme 3).

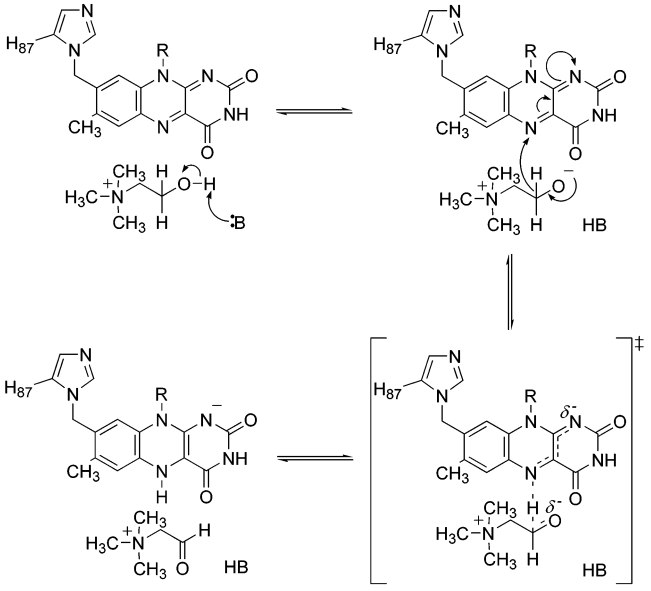
$$k_{cat} = \frac{k_3 k_7}{k_3 + k_7} \tag{9}$$

Evidence of chemical steps being rate limiting for the overall turnover of the enzyme with choline comes from both steady-state and rapid kinetics data. Indeed, a k_{cat} value of 55 s^{-1} can be estimated from the rate constants for flavin reduction determined with a stopped-flow spectrophotometer with choline and betaine aldehyde of 93 s^{-1} and 135 s^{-1}, respectively, agreeing well with the k_{cat} value of 60 s^{-1} obtained from steady-state measurements with choline as substrate. Independent evidence supporting chemical steps being solely rate limiting for the overall turnover of the enzyme with choline comes from the pH-independent $^Dk_{cat}$ value with 1,2-[$^2H_4$]-choline. Both chemical steps are sensitive to the isotopic composition of the substrate, but while the overall isotope effect for CH bond cleavage with 1,2-[$^2H_4$]-choline is contributed by primary and secondary isotope effects, that with betaine aldehyde formed upon oxidation of 1,2-[$^2H_4$]-choline is devoid of the α-secondary effect. It can be shown that, for an enzymatic reaction in which two steps are sensitive to the same type of isotope, the observed isotope effect is given by eq 10, the derivation of which is reported in the Supporting Information. A reasonable approximation of the $^Dk_3$ value, which is the intrinsic kinetic isotope effect for the enzymatic cleavage of the CH bond of choline, is provided by a value of ∼8.9 determined for the overall substrate deuterium kinetic isotope effect on the rate of flavin reduction determined with choline using a stopped-flow spectrophotometer. The intrinsic kinetic isotope effect for the enzymatic cleavage of the CH bond of betaine aldehyde, $^Dk_7$, can be estimated to be 1.24 times lower than that of choline due to the lack of an α-secondary effect, with a value of ∼7.2.[49] The observed $^Dk_{cat}$ value of ∼7.5 agrees reasonably well with the expected isotope effect of ∼8.2 that can be calculated by using eq 10, consistent with k_{cat} being limited only by the two chemical processes catalyzed by choline oxidase. Previous kinetic studies show that choline oxidase can accept betaine aldehyde as substrate.[23,36,39] Since both chemical steps of flavin reduction by choline and betaine aldehyde contribute to the overall turnover of the enzyme with choline, reduction of the flavin by betaine aldehyde should be solely responsible for the overall turnover with betaine aldehyde as substrate. This is

(48) Cleland, W. W. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1970; Vol. II; pp 1−65.

(49) Cleland, W. W. In *Methods in Enzymology*; Purich, D. L., Ed.; Academic Press: New York, 1980; Vol. 64; pp 104−125.

Scheme 4. Chemical Mechanism of Choline Oxidase for Oxidation of Choline at pH 10



indeed the case; the rate constant for reduction of the flavin by betaine aldehyde is ~135 s^{-1} agreeing well with the k_{cat} value of ~133 s^{-1} determined under steady-state conditions. The lack of effects of solvent viscosity on the k_{cat} value is further consistent with the overall turnover being limited by chemical steps, since no effect of solvent viscosity would be expected for the transfers of the hydride to the flavin. Finally, the $^{D_2O}k_{cat}$ value of unity suggests that the kinetic step in which betaine aldehyde is hydrated, which is associated with a solvent kinetic isotope effect of ~3.6 in nonenzymatic studies in solution,[50] does not contribute to the overall turnover.

$$^{D}k_{cat} = \frac{^{D}k_3 + ^{D}k_7\frac{k_3}{k_7}}{1 + \frac{k_3}{k_7}} \qquad (10)$$

The oxidation of choline catalyzed by choline oxidase occurs through the formation of an alkoxide species resulting from the removal of the substrate hydroxyl proton occurring before hydride transfer to the flavin (Scheme 4). Strong evidence for such a mechanism, in which the choline alkoxide proton is not in flight in the transition state for CH bond cleavage, comes from the substrate and solvent deuterium kinetic isotope effects determined on the rate of reduction of the flavin by choline, showing a substrate isotope effect of ~9 and a solvent isotope effect of unity. These data immediately rule out a concerted hydride transfer mechanism in which both the proton and the hydride are concomitantly in flight in the transition state, since if that were the case solvent isotope effects significantly larger than unity would have been observed. The rate of OH bond cleavage must be significantly faster than that of CH bond cleavage, as indicated by the lack of changes in the substrate isotope effect on the rate of flavin reduction by choline upon substituting aqueous with deuterated solvent. Consistent with this conclusion, a 9-fold decrease in the rate of CH bond cleavage, which is achieved by using deuterated choline as substrate for the enzyme, does not result in any significant change in the size of the solvent kinetic isotope effect on the rate of flavin reduction. The effects of substrate and solvent isoptomers on the k_{cat}/K_m value agree well with the measurements carried out on the stopped-flow spectrophotometer. An alternative mechanism in which a single electron is transferred to the flavin concomitantly with abstraction of the hydroxyl proton before hydrogen transfer to the flavin is unlikely since, based on the relative size of the substrate and solvent isotope effects, this would result in significant accumulation of a flavin radical species in turnover. However, single turnover data using a stopped-flow spectrophotometer indicate that the enzyme-bound flavin is reduced by choline directly to the hydroquinone state without formation of detectable transient flavin semiquinone species. In addition, previous biochemical and kinetic data strongly support the notion that in choline oxidase the flavin semiquinone is catalytically inert, thereby providing indirect evidence against a radical mechanism for catalysis.[36] An asynchronous transition state in which OH bond cleavage has occurred to a greater extent than CH bond cleavage was previously proposed for another member of the GMC oxidoreductase superfamily, methanol oxidase, based on solvent and primary deuterium kinetic isotope effect studies using a number of substituted alcohols.[31] In choline oxidase, the necessary stabilization of the incipient alkoxide species is provided by a protonated histidine residue, as recently suggested by biochemical, mechanistic, and spectroscopic studies of a mutant enzyme in which His_{466} was replaced by an alanine residue.[51]

Members of the GMC oxidoreductase superfamily share a similar overall fold and a highly conserved catalytic site, as shown by the X-ray crystallographic structures of cholesterol oxidase,[18−20] glucose oxidase,[14−16] and cellobiose dehydrogenase.[17,27] While the three-dimensional structure of choline oxidase is not available to date, both CD spectroscopic and computational modeling studies suggest that the overall fold and catalytic site of choline oxidase are similar to those of other GMC members,[36,51] suggesting a similar activation mechanism for the oxidation of their substrates. Previous studies with glucose oxidase and cholesterol oxidase showed that it is difficult to establish an unambiguous catalytic mechanism due to the chemical steps being not fully rate limiting in these two enzymes.[28,29] Nonetheless, glucose oxidase was reported to show a primary deuterium kinetic isotope effect on the k_{cat}/K_m value of 2 to 3 with 1-[^{2}H]-glucose as substrate and negligible solvent isotope effects.[28] Similarly, primary and solvent deuterium kinetic isotope effects of 2.2 and 1, respectively, were reported for the overall turnover of cholesterol oxidase with 3α-[^{2}H]-cholesterol.[29,30] With both enzymes, kinetic isotope effects resemble the pattern that was observed in the present study with choline oxidase, with detectable substrate isotope effects and negligible solvent isotope effects, in agreement with the alkoxide mechanism proposed in this study for choline oxidase.

Conclusion

The results of the mechanistic investigation with deuterated substrate and solvent presented in this study indicate that chemical steps are fully rate limiting for the overall turnover of

(50) Pocker, Y. *Proc. Chem. Soc.* **1960**, 17−18.

(51) Ghanem, M.; Gadda, G. *Biochemistry*, in press.

the flavoprotein choline oxidase. This has allowed the use of kinetic isotope effects as mechanistic probes for the elucidation of the catalytic mechanism for oxidation of the alcohol substrate by this flavin-dependent enzyme. The kinetic data showed that the removal of the hydroxyl proton and the transfer of the hydride from the substrate α-carbon to the flavin cofactor occur in a stepwise fashion, consistent with the formation of a transient alkoxide species. Recent structural and mechanistic data on flavocytochrome b_2, which is considered the paradigm for flavin-dependent enzymes that oxidize α-hydroxy acids, are most consistent with a hydride transfer mechanism occurring through the formation of an alkoxide species.[47,52] Thus, as both mechanistic and structural data accumulate, it appears that a common catalytic strategy in which the hydride transfer is facilitated by the enzyme-catalyzed formation of an alkoxide species has been adopted by flavin-dependent enzymes for the oxidation of both unpolarized and polarized alcohols.

(52) Sobrado, P.; Fitzpatrick, P. F. *Biochemistry* **2003**, *42*, 15208−15214.

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Supporting Information Available: The mathematic derivation of ${}^{D}k_{cat}$ for an enzymatic reaction in which two steps (i.e., k_3 and k_7) are sensitive to the same type of isotope (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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