Mechanistic Studies of Choline Oxidase with Betaine Aldehyde and Its Isosteric Analogue 3,3-Dimethylbutyraldehyde†

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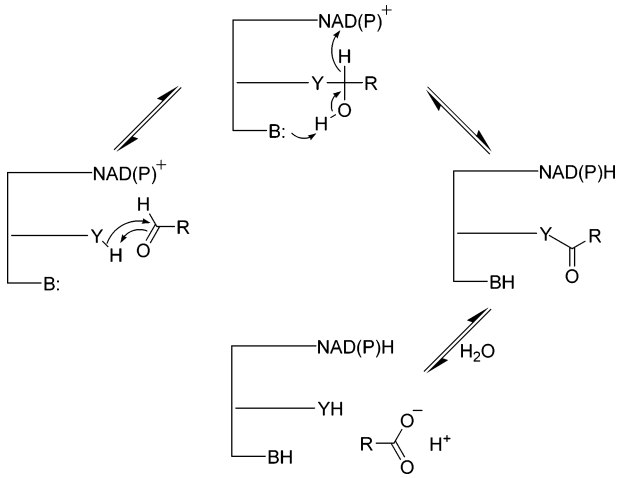
ABSTRACT: Choline oxidase catalyzes the four-electron oxidation of choline to glycine betaine via two sequential FAD-dependent reactions in which betaine aldehyde is formed as an intermediate. The chemical mechanism for the oxidation of choline catalyzed by choline oxidase was recently elucidated by using kinetic isotope effects [Fan, F., and Gadda, G. (2005) *J. Am. Chem. Soc. 127*, 2067−2074]. In this study, the oxidation of betaine aldehyde has been investigated by using spectroscopic and kinetic analyses with betaine aldehyde and its isosteric analogue 3,3-dimethylbutyraldehyde. The pH dependence of the k_{cat}/K_m and k_{cat} values with betaine aldehyde showed that a catalytic base with a pK_a of ∼6.7 is required for betaine aldehyde oxidation. Complete reduction of the enzyme-bound flavin was observed in a stopped-flow spectrophotometer upon anaerobic mixing with betaine aldehyde or choline at pH 8, with similar k_{red} values ≥48 s^{-1}. In contrast, only 10−26% of the enzyme-bound flavin was reduced by 3,3-dimethylbutyraldehyde between pH 6 and 10. Furthermore, this compound acted as a competitive inhibitor versus choline. NMR spectroscopic analyses indicated that betaine aldehyde exists predominantly (99%) as a diol form in aqueous solution. In contrast, the thermodynamic equilibrium for 3,3-dimethylbutyraldehyde favors the aldehyde (≥65%) over the hydrated form in the pH range from 6 to 10. The keto species of 3,3-dimethylbutyraldehyde is reactive toward enzymic nucleophiles, as suggested by the kinetic data with NAD^+-dependent yeast aldehyde dehydrogenase. The data presented suggest that choline oxidase utilizes the hydrated species of the aldehyde as substrate in a mechanism for aldehyde oxidation in which hydride transfer is triggered by an active site base.

The oxidation of aldehydes to their corresponding acids is an important biochemical reaction for maintaining normal cell functions, since aldehyde metabolites lead to cellular impairments due to their toxicity, mutagenicity, and carcinogenicity (*1*, *2*). In biological systems, enzymes that oxidize aldehydes have been shown to utilize nicotinamide nucleotides (*3*−*6*), molybdenum metal centers (*7*−*10*), and flavins (*11*−*16*) as essential cofactors for the reaction. Nicotinamide nucleotide-dependent enzymes are epitomized by the aldehyde dehydrogenase superfamily, which contains over 150 enzymes that differ in their substrate specificity and in the use of NAD^+ or $NADP^+$ as cofactors (*3*, *4*). Molybdenum-dependent enzymes include aldehyde oxidase and xanthine oxidase (*7*−*10*). Flavin-dependent enzymes comprise choline oxidase (*11*−*14*), choline dehydrogenase (*15*), and thiamin oxidase (*16*). Structural and mechanistic studies with aldehyde dehydrogenases convincingly suggested that the nicotinamide nucleotide-dependent oxidation of aldehydes occurs through the formation of a thiohemiacetal intermediate with an active site nucleophilic cysteine, followed by hydride ion transfer to the nicotinamide nucleotide (Scheme 1) (*17*−*27*). Hydrolysis of the resulting acyl-thioester intermediate then releases the acid product of the reaction from the enzyme active site. An alternative mechanism for the $NAD(P)^+$-

Scheme 1: Proposed Nucleophilic Mechanism for the Oxidation of Aldehydes Catalyzed by Aldehyde Dehydrogenase



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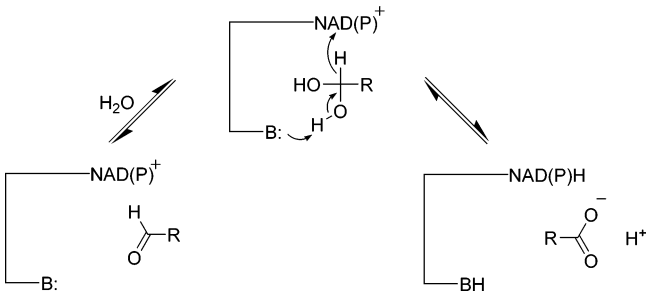
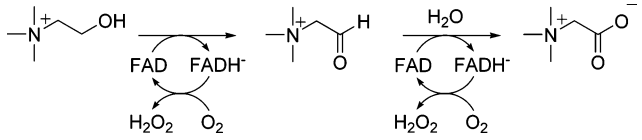
Scheme 2: Proposed *gem*-Diol Mechanism for the Oxidation of Histidinal Catalyzed by Histidinol Dehydrogenase



Scheme 3: Four-Electron Oxidation of Choline Catalyzed by Choline Oxidase



dependent oxidation of aldehydes, which was originally proposed for the oxidation of the histidinal intermediate in the reaction catalyzed by histidinol dehydrogenase (*28*, *29*) and for a mutant form of glyceraldehyde-3-phosphate dehydrogenase (*30*), is shown in Scheme 2. Here, the general base-catalyzed hydride transfer to the nicotinamide nucleotide would occur from the *gem*-diol form of the aldehyde substrate that is generated by the addition of a water molecule. The molybdenum-dependent oxidation of aldehydes has been proposed to proceed by transfer of a metal-activated molybdenum-bound water molecule to the carbonyl carbon of the substrate in concert with hydride transfer to the sulfido group, followed by cleavage of the substrate−molybdenum intermediate through electron transfer to other redox-active centers and release of product by hydroxide from solvent (*31*−*33*). To date, studies that address the mechanism of aldehyde oxidation catalyzed by flavin-dependent enzymes have not been reported.

Choline oxidase (EC 1.1.3.17) catalyzes the four-electron oxidation of choline to glycine betaine via two sequential FAD-dependent reactions in which betaine aldehyde (BA)[1] is formed as an intermediate (Scheme 3) (*11*, *13*, *14*) and is capable of using betaine aldehyde as substrate (*13*, *14*). This reaction is of considerable interest for medical and biotechnological applications, since intracellular accumulation of glycine betaine allows normal cell function under conditions of hyperosmotic and temperature stress in pathogenic bacteria (*34*−*37*) and transgenic plants (*38*−*43*). Consequently, the study of choline oxidase has potential for the development of therapeutic agents that inhibit glycine betaine biosynthesis and render pathogenic bacteria susceptible to either conventional treatments or the immune system and for the engineering of drought and temperature resistance in economically relevant crops. From a fundamental standpoint, choline oxidase offers a unique opportunity to elucidate the mechanism of oxidation of alcohols and aldehydes within the same active site of a flavin-dependent enzyme.

The chemical mechanism for the oxidation of choline catalyzed by choline oxidase has been recently elucidated using kinetic isotope effects and is consistent with the quantum mechanical transfer of a hydride from the substrate α-carbon to the enzyme-bound flavin cofactor occurring from an activated alkoxide form of choline (*44*−*46*). Such an alkoxide species has been proposed to form by the action of an active site base with a pK_a of ∼7.5 (*47*−*49*) and to be electrostatically stabilized in the enzyme active site by the imidazolium side chain of His_{466} (*45*). The oxidation of betaine aldehyde likely requires an activated form of the substrate, either through a nucleophilic mechanism or through the formation of a *gem*-diol species. In the present study, the aldehyde oxidation by choline oxidase has been studied using betaine aldehyde and its isosteric analogue, 3,3-dimethylbutyraldehyde (DMBA), in combination with spectroscopic and kinetic analyses in the steady state and pre steady state. The results of these studies provide insights into the mechanism for the oxidation of aldehydes catalyzed by choline oxidase and represent the first instance in which a chemical mechanism for aldehyde oxidation involving a flavin cofactor is presented.

EXPERIMENTAL PROCEDURES

Materials. Choline chloride was from ICN. Betaine aldehyde chloride and aldehyde dehydrogenase from baker's yeast were from Sigma. 3,3-Dimethylbutyraldehyde (95%) was from Aldrich. All other reagents were of the highest purity commercially available.

Enzyme Purification. Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified to homogeneity as described by Fan et al. (*14*). Fully oxidized enzyme was prepared as described by Gadda et al. (*48*).

Enzyme Assays. Enzyme activity was measured polarographically by monitoring the rate of oxygen consumption with a Hansatech oxygen electrode thermostated at 25 °C. The reactions were initiated by adding 5 μL of choline oxidase to a final concentration of ∼0.1 μM in a 1 mL reaction mixture. Unless otherwise stated, air-saturated 50 mM sodium pyrophosphate was used as reaction buffer for all experiments except for pH 7 where 50 mM potassium phosphate was used. Product inhibition studies were carried out by varying the concentrations of 3,3-dimethylbutyraldehyde (from 0 to 30 mM) and choline (from 0.5 to 10 mM), at pH 8. The pH-dependence studies were carried out in a reaction mixture that contained from 0.05 to 40 mM betaine aldehyde, over the pH range from 5 to 10. In all cases, enzyme activity is expressed per active site flavin content (*49*). Rapid kinetics were 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 at 454 nm in the absorbance resulting from reduction of the enzyme-bound flavin species upon mixing the enzyme and substrate. The enzyme solution in 20 mM Tris-HCl, pH 8, 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 glucose

[1] Abbreviations: BA, betaine aldehyde; DMBA, 3,3-dimethylbutyraldehyde.

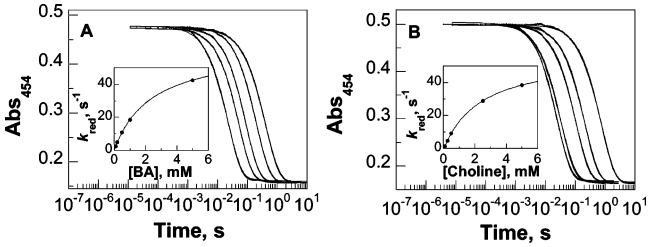


FIGURE 1: Anaerobic reductions of choline oxidase with betaine aldehyde (panel A) and choline (panel B). Assays were carried out in a stopped-flow spectrophotometer with 0.1, 0.25, 0.5, 2.5, and 5 mM choline or 0.1, 0.2, 0.5, 1, and 5 mM betaine aldehyde in 20 mM Tris-HCl, pH 8, at 25 °C. All traces were fit to eq 1. Insets: Observed rates of flavin reduction as a function of substrate concentration. Data were fit to eq 2.

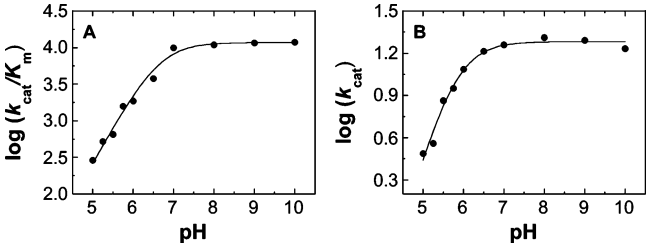


FIGURE 2: pH dependence of k_{cat}/K_m (M^{-1} s^{-1}, panel A) and k_{cat} (s^{-1}, panel B) values at atmospheric oxygen with betaine aldehyde as substrate. Activity assays of choline oxidase were performed in 50 mM buffer at 25 °C. Data were fit to eq 3.

oxidase. The organic substrate (~2 mL) was dissolved in 20 mM Tris-HCl, pH 8, and then degassed by flushing oxygen-free argon for at least 15 min before mounting onto the stopped-flow spectrophotometer. The enzyme was mixed anaerobically with an equal volume of substrate, yielding a reaction mixture containing ~40 μM choline oxidase and 0.05−5 mM choline or betaine aldehyde, in 20 mM Tris-HCl, pH 8. For each concentration of the substrate, reduction was observed in triplicate, and the average value for the observed rate constants was calculated. Typically, measurements differed by less than 5%. Reduction of choline oxidase by 3,3-dimethylbutyraldehyde or betaine aldehyde was carried out spectrophotometrically by mixing the enzyme and the ligand from the sidearm of an anaerobic cell, after 23 cycles of alternatively applying vacuum and argon, in 20 mM Tris-HCl, pH 8, at 15 °C. The enzymatic activity of aldehyde dehydrogenase from baker's yeast was measured spectrophotometrically by monitoring the increase in absorbance at 340 nm (with $\epsilon_{340} = 6.22$ mM^{-1} cm^{-1}) resulting from the formation of NADH associated with the enzymatic oxidation of the organic substrate in 200 mM KCl, 50 mM β-mercaptoethanol, 1 mM EDTA, and 100 mM Tris-HCl, pH 8, at 25 °C. The final concentration of NAD^+ was kept constant at 0.5 mM, whereas those of 3,3-dimethylbutyraldehyde and betaine aldehyde were in the range from 2 to 20 mM and from 5 to 40 mM, respectively. One unit of enzymatic activity corresponds to the formation of 1 μmol of NADH/min.

NMR Spectroscopy. Samples for 1D proton NMR spectroscopic analyses were prepared at a final concentration of 50 mM in 99.9% D_2O, with the pD adjusted to the desired values by using DCl or NaOD. NMR spectra were acquired at 298 K on a Bruker Avance 600 NMR spectrometer using a 5 mm QXI inverse probehead with a *z* gradient. The residual water peak was suppressed by presaturation during the 4 s relaxation delay. Data were processed and integrated using xwinnmr 3.5. No changes in the NMR spectra were observed when the analyses were repeated after 48 h.

Data Analysis. Data were fit with KaleidaGraph software (Synergy Software, Reading, PA) and Enzfitter software (Biosoft, Cambridge, U.K.). Stopped-flow traces were fit to eq 1, which describes a case for single exponential decay, in which k_{obs} represents the first-order rate constant for anaerobic substrate reduction of the enzyme-bound flavin, *t* is time, A_t is the value of absorbance at 454 nm, *A* is the amplitude of the total change, and A_∞ is the absorbance at infinite time. The pre-steady-state kinetic parameters were determined by using eq 2, where k_{obs} is the observed rate for the reduction of the enzyme-bound flavin, k_{red} is the limiting rate of flavin reduction at saturated substrate concentration, and K_d is the dissociation constant for substrate binding. The pH dependences of steady-state kinetic parameters were determined by fitting initial rate data to eq 3, which describes a curve with slope of +1 and a plateau region at high pH. *C* is the pH-independent value of the kinetic parameter of interest. Product inhibition studies with 3,3-dimethylbutyraldehyde as inhibitor were fit to eq 4, which describes the competitive inhibition pattern of 3,3-dimethylbutyraldehyde versus choline. *I* is the concentration of 3,3-dimethylbutyraldehyde, and K_{is} is the inhibition constant for the slope effect.

$$A_t = A\exp(-k_{obs}t) + A_\infty \quad (1)$$

$$k_{obs} = k_{red}A/(K_d + A) \quad (2)$$

$$\log Y = \log\left[\frac{C}{1 + (10^{-pH}/10^{-pK_a})}\right] \quad (3)$$

$$\frac{v}{e} = \frac{k_{cat}A}{K_a[1 + (I/K_{is})] + A} \quad (4)$$

RESULTS

As the first step toward the mechanistic characterization of aldehyde oxidation by choline oxidase, the rate of flavin reduction was determined by anaerobic mixing of the enzyme with betaine aldehyde by monitoring the changes at 454 nm in a stopped-flow spectrophotometer at pH 8. As expected from previous results at pH 10 (*44*), complete reduction of the enzyme-bound flavin was observed without detection of transient species (Figure 1), with a k_{red} value of 63 ± 0.3 s^{-1} and a K_d value of 2.3 ± 0.1 mM. When betaine aldehyde was substituted with choline as substrate, the k_{red} and K_d values were 48 ± 0.3 s^{-1} and 1.8 ± 0.3 mM (Figure 1), consistent with similar reactivity and binding affinity of choline oxidase toward betaine aldehyde and choline.

A previous study established that, with betaine aldehyde, no ionizable groups with pK_a between 6.5 and 10 participate in the oxidative half-reaction in which the enzyme-bound flavin reacts with O_2 (*49*). Accordingly, the determination of pK_a values for groups involved in the oxidation of betaine aldehyde could be carried out at a fixed concentration of O_2 by measuring initial rates of reaction at varying concentrations of betaine aldehyde in air-saturated buffer over the pH range from 5 to 10. As shown in Figure 2, both the k_{cat}/K_m and k_{cat} values for betaine aldehyde increased with increasing

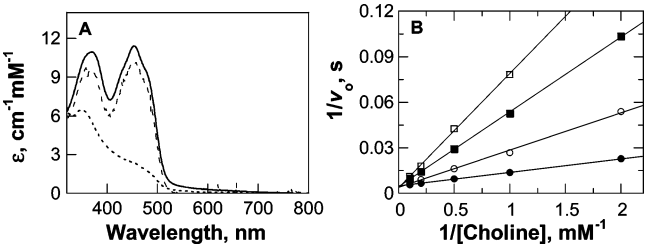
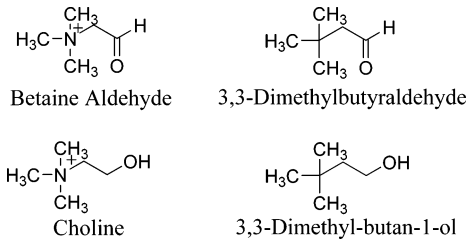


FIGURE 3: Spectrophotometric and kinetic studies of choline oxidase with 3,3-dimethylbutyraldehyde. Panel A: Reduction of the enzyme-bound flavin with 3,3-dimethylbutyraldehyde. Choline oxidase at a concentration of 80 μM in flavin content in 20 mM Tris-HCl, pH 8, at 15 °C, before (solid curve) and ~15 s after anaerobic addition of 10 mM 3,3-dimethylbutyraldehyde (dotted curve) or 4.5 mM betaine aldehyde (dashed curve). Panel B: Kinetic studies for inhibition of choline oxidase with 3,3-dimethylbutyraldehyde. Initial rates were measured in air-saturated 50 mM sodium pyrophosphate, pH 8, at 25 °C. 3,3-Dimethylbutyraldehyde concentrations were 0 (●), 10 (○), 20 (■), and 30 mM (□). The lines represent a global fit of the data to eq 4.

Chart 1



pH and reached limiting values at high pH, with apparent pK_a values of 6.7 ± 0.1 and 5.7 ± 0.1, respectively. These data suggest the involvement of an unprotonated group in the reductive half-reaction in which betaine aldehyde is oxidized to glycine betaine.

When choline is substituted with its isosteric analogue 3,3-dimethylbutan-1-ol (Chart 1) as substrate for choline oxidase, a 10-fold decrease in the overall turnover number of the enzyme is observed (*47*−*49*), suggesting that the positive charge on the trimethylammonium moiety of the substrate is important but not essential for catalysis. A similar decrease in the activity of choline oxidase would therefore be expected when 3,3-dimethylbutyraldehyde is used as substrate for the enzyme instead of betaine aldehyde. However, the anaerobic incubation of choline oxidase with 10 mM 3,3-dimethylbutyraldehyde at pH 8 resulted in only 13% of the flavin being reduced within the mixing time (≤15 s) (Figure 3A). Similar results were obtained at pH 6 and 10, with only 26% and 10% of the enzyme-bound flavin being reduced, respectively (see Figure S1 in Supporting Information for the data at pH 6). As expected from pH studies with choline (*47*−*49*) and betaine aldehyde (this study) as substrate for the enzyme, the rate of flavin reduction at pH 6 was slower than that at higher pH values, with a k_{obs} of ~0.04 s^{-1} with 10 mM 3,3-dimethylbutyraldehyde[2] (see Figure S1 in Supporting Information). In contrast, the enzyme-bound flavin was immediately reduced within the mixing time by anaerobic incubation of the enzyme with betaine aldehyde (Figure 3A), consistent with the rapid kinetic data showing a k_{red} of ~60 s^{-1}. To evaluate whether 3,3-dimethylbutyraldehyde might act as an inhibitor for choline oxidase, initial rates of reaction were measured at different fixed concentrations of 3,3-dimethylbutyraldehyde and varying concentrations of choline in air-saturated 50 mM sodium pyrophosphate, pH 8. As shown in Figure 3B, a pattern with lines intersecting on the *y*-axis was determined in a double reciprocal plot of 1/rate versus 1/[choline] when the concentration of 3,3-dimethylbutyraldehyde was varied, consistent with 3,3-dimethylbutyraldehyde being a competitive inhibitor versus choline. An apparent K_{is} value of 7.3 ± 0.6 mM for 3,3-dimethylbutyraldehyde binding to the enzyme was determined from these studies, in agreement with K_{is} values previously determined for binding of a number of substituted amines to choline oxidase (*48*). The dual kinetic behavior of 3,3-dimethylbutyraldehyde, which acts as a competitive inhibitor with respect to choline and as a poor substrate for the enzyme, along with the spectral changes in the visible absorbance spectrum of the enzyme-bound flavin that are observed in the presence of the aldehyde, suggests that 3,3-dimethylbutyraldehyde binds at the active site of choline oxidase.

Aldehydes in aqueous solution are known to exist in equilibrium with their hydrated species (*50*). Consequently, we have used NMR spectroscopy to determine the hydration ratio of betaine aldehyde and 3,3-dimethylbutyraldehyde, to investigate whether different diol/aldehyde ratios may be responsible for the different reactivity of choline oxidase with these aldehydes. At pD 8.5, the chemical shifts for the α-carbon-linked proton of betaine aldehyde were observed at 5.51 and 9.46 ppm, corresponding to the diol and keto species, respectively. A [diol]/[aldehyde] ratio of ~99 was calculated by integrating the peak areas, indicating that betaine aldehyde is predominantly hydrated in aqueous solution. In contrast, the integration of the peaks for the α-carbon-linked proton of 3,3-dimethylbutyraldehyde, which showed chemical shifts at 5.10 and 9.74 ppm for the diol and keto species, respectively, yielded a [diol]/[aldehyde] ratio of ~0.3, suggesting that 3,3-dimethylbutyraldehyde exists in aqueous solution at pD 8.5 as a mixture of ~23% hydrated and ~77% nonhydrated species. When the analysis was extended over the pD range from 6 to 12, no significant changes in the [diol]/[aldehyde] ratio were observed with betaine aldehyde, whereas the relative amount of diol species of 3,3-dimethylbutyraldehyde decreased slightly with increasing pD (Table 1).

To establish the reactivity of 3,3-dimethylbutyraldehyde in the presence of enzymic nucleophiles, 3,3-dimethylbutyraldehyde was tested as a substrate for baker's yeast aldehyde dehydrogenase, a member of the nicotinamide nucleotide-dependent aldehyde dehydrogenase superfamily for which a nucleophilic mechanism for aldehyde oxidation was convincingly established (*17*−*27*). Aldehyde dehydrogenase activity was determined spectrophotometrically by monitoring the initial rate of increase in absorbance at 340

[2] After the rapid reduction of the enzyme-bound flavin by 3,3-dimethylbutyraldehyde with a k_{obs} value of ~0.04 s^{-1}, a further decrease of 3% in the absorbance at 456 nm was observed over 30 min of incubation, consistent with the presence of a second phase of flavin reduction that is significantly slower than the initial rapid reaction. Slow phases of flavin reduction were observed also at pH 8 and 10, accounting for further decreases of 5% and 17% in the peak at 452 nm over 30 min of incubation, respectively. Although this kinetic behavior was not investigated further in this study, it is likely that the second phase of enzyme reduction reflects the slow conversion of the aldehyde form of 3,3-dimethylbutyraldehyde to the diol species that acts as the substrate for the enzyme.

Table 1: NMR Determination of Hydration Ratio of Betaine Aldehyde and 3,3-Dimethylbutyraldehyde in Aqueous Solution[a]

pD	betaine aldehyde [diol]/[aldehyde]	3,3-dimethylbutyraldehyde [diol]/[aldehyde]
5.9	nd[b]	0.42
6.9	99	nd
7.3	99	0.35
8.5	99	0.27
9.5	99	0.22
10.7	99	0.20
12.1	99	0.14

[a] Conditions: 50 mM aldehyde, dissolved in 99.9% D_2O, at 25 °C. [b] nd, not determined.

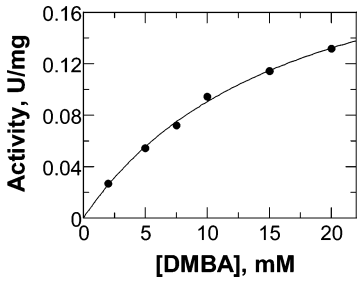


FIGURE 4: Kinetics of baker's yeast aldehyde dehydrogenase with 3,3-dimethylbutyraldehyde (DMBA) as substrate. Assays were carried out with 3,3-dimethylbutyraldehyde as substrate in 200 mM KCl, 0.5 mM NAD^+, 50 mM β-mercaptoethanol, 1 mM EDTA, and 100 mM Tris-HCl, pH 8, at 25 °C. Data were fit into the Michaelis−Menten equation for one substrate.

nm that results from the formation of NADH that is associated with the enzymatic oxidation of the organic substrate. As shown in Figure 4, 3,3-dimethylbutyraldehyde acted as substrate for aldehyde dehydrogenase, following typical saturation kinetics with an apparent V_{max}/K_m value[3] of 14400 ± 10 units mM^{-1} (mg of enzyme)$^{-1}$. In contrast, no enzymatic activity was observed with concentrations of betaine aldehyde in the range from 5 to 40 mM, consistent with previous analyses showing that yeast aldehyde dehydrogenase is unable to oxidize hydrated aldehydes (*51*). These data clearly indicate that in the presence of a suitable enzymic nucleophile 3,3-dimethylbutyraldehyde is reactive.

DISCUSSION

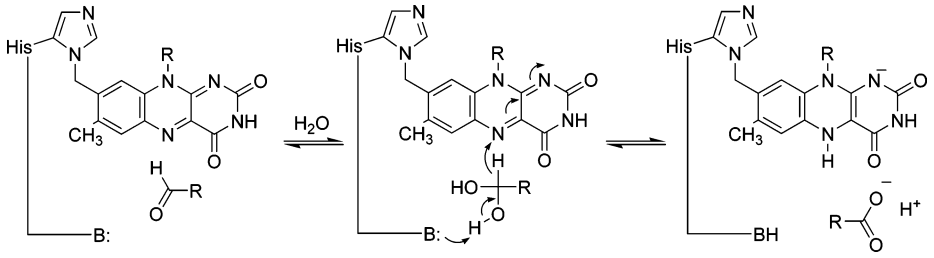
The oxidation of choline to glycine betaine catalyzed by choline oxidase occurs through two sequential flavin-linked hydride transfers in which betaine aldehyde is formed as an enzyme-bound intermediate (*12*−*14*). The first oxidation reaction in which choline is oxidized to betaine aldehyde was recently characterized by using kinetic isotope effects and is consistent with the quantum mechanical transfer of a hydride ion from an activated choline−alkoxide species that is formed in the enzyme−substrate complex (*44*, *46*). An active site residue with a pK_a of ∼7.5 has been proposed to act as a base for the activation of choline to yield the alkoxide species (*47*−*49*). The choline−alkoxide transient species is then stabilized in the enzyme active site through electrostatic interaction with the imidazolium side chain of His_{466} (*45*). The second oxidation reaction in which betaine aldehyde is oxidized to glycine betaine might also require an activated form of the substrate. Such an aldehyde substrate activation can occur either through the formation of an acyl substrate−enzyme intermediate, as in the nucleophilic mechanism proposed for the nicotinamide nucleotide-dependent aldehyde dehydrogenases (Scheme 1) (*17*−*27*), or by the addition of a water molecule to yield a *gem*-diol, as in the mechanism proposed for histidinol dehydrogenase (Scheme 2) (*28*, *29*). A distinguishing feature of these mechanisms lies in the hydration states of the aldehyde substrates. The nonhydrated species is required for the nucleophilic attack that results in the acyl substrate−enzyme intermediate, whereas the hydrated species is required for the base-catalyzed *gem*-diol mechanism. The NMR spectroscopic analyses presented herein have shown that in aqueous solution betaine aldehyde and its isosteric analogue 3,3-dimethylbutyraldehyde are hydrated to different extents. Since choline oxidase was recently shown to be capable of oxidizing substrate analogues devoid of a positive charge on the trimethylammonium group (*48*), here we have used betaine aldehyde and 3,3-dimethylbutyraldehyde as probes to gain insights on the chemical mechanism for the second oxidation reaction catalyzed by choline oxidase.

In aqueous solution, betaine aldehyde exists predominantly (99%) in the hydrated form, as suggested by the NMR spectroscopic analysis reported in this study. In contrast, the hydration equilibrium for 3,3-dimethylbutyraldehyde favors the nonhydrated species, with only ∼20% of this aldehyde being in the hydrated form at pH 8. The larger hydrated to nonhydrated ratio observed for betaine aldehyde compared to 3,3-dimethylbutyraldehyde likely stems from the presence of the positively charged trimethylammonium group, which would stabilize the partial negative charges of the hydroxyl groups in the hydrated aldehyde species.

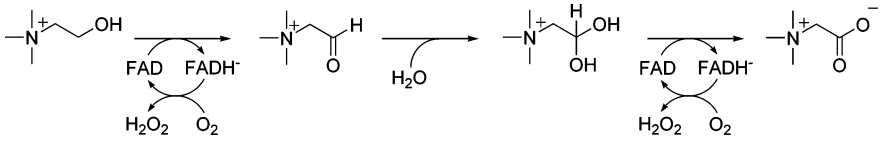
The hydrated aldehyde form of betaine aldehyde is required for the second oxidation reaction catalyzed by choline oxidase in which betaine aldehyde is converted to glycine betaine, consistent with the *gem*-diol mechanism of Scheme 4. Strong evidence for such a mechanism comes from the kinetic and spectrophotometric data with betaine aldehyde and 3,3-dimethylbutyraldehyde as substrates for the enzyme. Indeed, betaine aldehyde, which is predominantly hydrated in solution, is a good substrate for choline oxidase, with a rate of reduction of the enzyme-bound flavin that is slightly faster than the rate determined with choline both at pH 8 (this study) and at pH 10 (*44*). As expected from the similarity in the chemical structures of choline and hydrated betaine aldehyde, choline oxidase shows similar binding affinities for these substrates, with K_d values of ∼2 mM at pH 8 (this study) and ∼0.4 mM at pH 10 (*44*). In contrast, with 3,3-dimethylbutyraldehyde only the fraction of enzyme that binds the hydrated form (∼20%) is anaerobically reduced between pH 6 and 10, whereas the majority of the enzyme does not react because the aldehyde form of 3,3-dimethylbutyraldehyde acts as a competitive inhibitor of the enzyme with respect to choline, as suggested by the inhibition studies.[4] In principle, a nucleophilic mechanism for aldehyde oxidation in which choline oxidase reacts poorly with 3,3-

[3] The reason why only the V_{max}/K_m value is reported here is that substrate inhibition at concentrations larger than 30 mM did not allow saturation of aldehyde dehydrogenase with 3,3-dimethylbutyraldehyde to be reached (data not shown), thereby preventing accurate estimates of the V_{max} and K_m values. Since the rationale for this experiment was to establish whether 3,3-dimethylbutyraldehyde reacts in the presence of enzymic nucleophiles, which has been established by the data presented here, no further investigation of the kinetics of oxidation of 3,3-dimethylbutyraldehyde by aldehyde dehydrogenase was carried out.

Scheme 4: Proposed Mechanism for the Oxidation of Betaine Aldehyde Catalyzed by Choline Oxidase



Scheme 5: Hydration of Betaine Aldehyde during Turnover of Choline Oxidase with Choline



dimethylbutyraldehyde as compared to betaine aldehyde would still be possible if 3,3-dimethylbutyraldehyde were inherently unreactive toward enzymic nucleophiles. However, the observation that 3,3-dimethylbutyraldehyde is a good substrate for aldehyde dehydrogenase, for which a nucleophilic mechanism for aldehyde oxidation has been unequivocally established (*17*−*27*), immediately rules out an inherent unreactivity of this aldehyde toward suitable enzymic nucleophiles as a rationale to explain the poor reactivity of choline oxidase with 3,3-dimethylbutyraldehyde. Taken together, the hydration ratios of betaine aldehyde and 3,3-dimethylbutyraldehyde, the structural similarities, and comparable rates of flavin reduction of betaine aldehyde and choline, as well as the poor reactivity of the enzyme with 3,3-dimethylbutyraldehyde, are best explained by a mechanism of aldehyde oxidation in which the hydrated aldehyde acts as substrate for the enzyme.

A group with an apparent pK_a of ∼6.7 must be unprotonated for the oxidation of betaine aldehyde catalyzed by choline oxidase, as suggested by the pH profile of the apparent k_{cat}/K_m value with betaine aldehyde as substrate. The involvement of this group in chemical steps of proton transfer rather than substrate binding is supported by the observation that the requirement for an unprotonated group is also seen in the k_{cat} pH profile (*52*). Previous kinetic data established the lack of ionizable groups with pK_a between 6.5 and 10 in the oxidative half-reaction in which the reduced flavin is oxidized by dioxygen during catalysis with betaine aldehyde (*49*), consistent with the group with a pK_a value of ∼6.7 being involved in the reductive half-reaction in which betaine aldehyde is oxidized to glycine betaine. A likely role for this group is to act as a catalytic base by abstracting a hydroxyl proton from hydrated betaine aldehyde to trigger the transfer of a hydride ion from the substrate α-carbon to the enzyme-bound flavin, as illustrated in Scheme 4. A similar role for an active site residue was recently proposed for the oxidation of choline catalyzed by the choline oxidase (*44*). Because both oxidation reactions occur at the same active site, it is reasonable to expect that the same amino acid residue be involved in the oxidation of both organic molecules.

The pK_a value of 5.7 seen in the k_{cat} pH profile is likely due to the same residue responsible for the pK_a value of 6.7 observed in the k_{cat}/K_m pH profile. Its pK_a value would be perturbed in the k_{cat} pH profile by the presence of slower kinetic step(s) occurring after the proton transfer step (*52*): specifically, the oxidation of the enzyme-bound flavin under atmospheric oxygen conditions. Previous studies established that, at saturating concentrations of O_2, flavin reduction is solely rate-limiting for the overall turnover number with betaine aldehyde, with a limiting value of 135 s^{-1} at high pH (*44*). Since the k_{cat}/K_{O_2} value with betaine aldehyde is ∼6 × 10^4 M^{-1} s^{-1} (*49*), the rate constant for oxidation of the enzyme-bound flavin under atmospheric conditions, i.e., at a concentration of oxygen of 0.25 mM at 25 °C, is therefore expected to be ∼15 s^{-1}. Consequently, the apparent pK_a value observed in the k_{cat} pH profile would be perturbed

[4] The observed rate of flavin reduction of ∼0.04 s^{-1} with 10 mM 3,3-dimethylbutyraldhyde at pH 6 is comparable to the value that can be estimated upon taking into account the lower amount of hydrated aldehyde available for the oxidation reaction as compared to betaine aldehyde, the low reactivity of the enzyme with substrates devoid of a positively charged amino moiety, and the inhibition of the enzyme by the nonhydrated form of the aldehyde. Indeed, our kinetic studies showed that with the choline analogue devoid of a positive charge, 3,3-dimethylbutan-1-ol, choline oxidase has lower k_{cat} values and higher K_m values with respect to choline as substrate at pH 8 (*48*). Similar results were obtained for lower pH values, with a k_{cat} value of 0.4 s^{-1} and a K_m of 11 mM as compared to 9 s^{-1} and 0.9 mM with choline as substrate at pH 6.5 (Table S1 in Supporting Information). Because the enzyme has similar k_{red} and K_d values with betaine aldehyde and choline, similar binding affinities and reactivities are predicted with 3,3-dimethylbutan-1-ol and 3,3-dimethylbutyraldehyde. Thus, the estimated rate of flavin reduction with 10 mM 3,3-dimethylbutyraldehyde, of which ∼30% is hydrated and acts as substrate and ∼70% is nonhydrated and acts as a competitive inhibitor, can be estimated to be ∼0.1 s^{-1} by using $k_{obs} = (Sk_{cat})/[S + K_m(1 + I/K_{is})]$, where k_{cat} is ∼0.4 s^{-1}, S is 3 mM, the concentration of hydrated aldehyde that acts as substrate, I is 7 mM, the concentration of nonhydrated aldehyde that acts as inhibitor, and K_m and K_{is} are 11 mM and 7 mM, the dissociation constant estimated for 3,3-dimetrylbutyraldehyde and the inhibition constant for the aldehyde species, respectively.

[5] Substrate and solvent kinetic isotope effect studies with deuterated choline have established that in the oxidation of choline catalyzed by choline oxidase the cleavage of the OH bond occurs prior of cleavage of the CH bond (*44*). While in that study it was established that cleavage of the CH bond in the oxidation of betaine aldehyde is fully rate limiting for the overall turnover of the enzyme when betaine aldehyde is the substrate, no information is available that establishes the relative timing for the cleavages of the OH and CH bonds with the aldehyde substrate. Consequently, while the basic features for the alcohol and aldehyde oxidation reactions are similar, in that they both proceed via hydride transfer from the substrate α-carbon to the N(5) flavin atom, it remains to be established whether the cleavages of the OH and CH bonds of the aldehyde substrate occur asynchronously as in the case of choline.

to lower values by 1 pH unit, as experimentally observed, from the relationship shown in eq 5, where k_{red} and k_{ox} are the rate constants for the chemical steps of flavin reduction and oxidation with values of 135 s^{-1} and 15 s^{-1}, respectively (*52*). A similar pattern where the pK_a value determined from the k_{cat} pH profiles at atmospheric O_2 was perturbed with respect to that determined from the k_{cat}/K_m pH profiles was recently reported with choline and its analogues as substrate for choline oxidase (*48*, *49*).

$$\Delta pK_a = \log[1 + (k_{red}/k_{ox})] \quad (5)$$

Previous kinetic and biochemical studies established that, during turnover of choline oxidase with choline, betaine aldehyde is further oxidized to glycine betaine before dissociating from the active site of the enzyme (*49*), consistent with two sequential flavin-linked reactions in which choline is first oxidized to betaine aldehyde and betaine aldehyde is subsequently oxidized to glycine betaine. Since the product of the first oxidation reaction is the keto species of the aldehyde intermediate and the substrate of the second oxidation reaction is the diol form of the aldehyde intermediate, a hydration step must necessarily occur during turnover of the enzyme with choline after formation of betaine aldehyde and prior of the second oxidation reaction (Scheme 5). Such hydration of the enzyme-bound betaine aldehyde intermediate is kinetically fast, as suggested by recent kinetic data showing that the overall turnover number with choline is not altered in deuterated solvent and is limited solely by the chemical steps of hydride transfer (*44*).

In conclusion, the results of the mechanistic investigation of choline oxidase with betaine aldehyde and its isosteric analogue 3,3-dimethylbutyraldehyde lacking the positively charged amino moiety presented in this study are best explained with a mechanism for aldehyde oxidation in which a hydrated form of the aldehyde substrate is required for catalysis. Aldehyde oxidation then occurs via a base-catalyzed transfer of a hydride ion from the organic substrate to the enzyme-bound flavin, with a mechanism that, in its fundamental form, is similar to that proposed for the oxidation of choline catalyzed by same enzyme[5] (*44*). Thus, it appears that nature has elegantly solved the difficult task of catalyzing two sequential flavin-linked oxidation reactions of an alcohol and an aldehyde within the same enzyme active site in the least energetically expensive fashion by utilizing similar catalytic strategies.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 showing selected spectra illustrating the anaerobic reduction of choline oxidase with 10 mM 3,3-dimethylbutyraldehyde as substrate at pH 6 and Table S1 summarizing the steady-state kinetic data with choline or 3,3-dimethylbutan-1-ol as substrate for choline oxidase at pH 6.5. This material is available free of charge via the Internet at http://pubs.acs.org.

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