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Relative Timing of Hydrogen and Proton Transfers in the Reaction of Flavin Oxidation Catalyzed by Choline Oxidase

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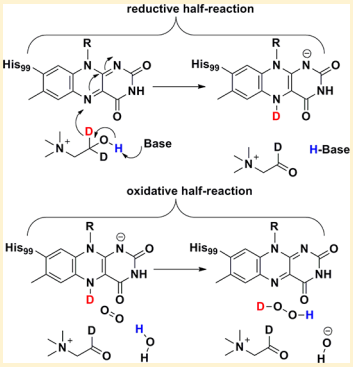
ABSTRACT: The oxidation of the reduced flavin in choline oxidase was investigated with pH, solvent viscosity, and kinetic isotope effects (KIEs) in steady-state kinetics and time-resolved absorbance spectroscopy of the oxidative half-reaction in a stopped-flow spectrophotometer. Both the effects of isotopic substitution on the KIEs and the multiple KIEs suggest a mechanism for flavin oxidation in which the H atom from the reduced flavin and a H^+ from the solvent or a solvent exchangeable site are transferred in the same kinetic step. Stopped-flow kinetic data demonstrate flavin oxidation without stabilization of flavin-derived species. Solvent viscosity effects establish an isomerization of the reduced enzyme. These results allow us to rule out mechanisms for flavin oxidation in which C4a-peroxy and -hydroperoxy flavin intermediates accumulate to detectable levels in the reaction of flavin oxidation catalyzed by choline oxidase. A mechanism of flavin oxidation that directly results in the formation of oxidized flavin and hydrogen peroxide without stabilization of reaction intermediates is consistent with the data presented.



Flavin-dependent oxidases and monooxygenases react rapidly with dioxygen (O_2), with second-order rate constants of 10^4–10^6 M^{-1} s^{-1}.[1,2] The former yield H_2O_2, while the latter yield H_2O and a hydroxylated organic product.[1,2] Because of the diradical nature of the stable form of O_2 in the atmosphere, the direct transfer of an electron pair from the reduced flavin to O_2 is spin forbidden and cannot occur.[3−5] Hence, O_2 reduction proceeds through two single-electron transfers that generate a highly reactive flavosemiquinone/$O_2^{-\bullet}$ radical pair (Scheme 1, step a).[1−8] A reaction intermediate with spectroscopic features similar to those of a flavosemiquinone was recently reported in the oxidative half-reaction of glycolate oxidase conducted in D_2O.[9] Reduction of O_2 in monooxygenases typically reveals detectable C4a-hydroperoxy and -peroxy flavin intermediates in rapid kinetic studies (Scheme 1, routes a-b-g and a-d-h, respectively).[1,10,11] With the exception of pyranose 2-oxidase[12,13] and a mutant form of NADH oxidase,[14] those intermediates are not typically observed in oxidases, where rapid kinetics show monophasic oxidations of the reduced flavin.[1,2] Studies of glucose oxidase using ^{18}O kinetic isotope effects (KIEs), the enzyme reconstituted with flavin analogues, and temperature effects support the notion that O_2 reduction occurs without formation of flavin intermediates (Scheme 1, route a-c).[15] Mutagenesis demonstrated that a positively charged histidine in glucose oxidase, H516, is the main site for O_2 activation through electrostatic catalysis.[4,16]

Irrespective of the mechanism, flavin oxidation by flavoprotein oxidases requires the transfer of one electron, a hydrogen (H) from the reduced flavin N5 atom (Scheme 1, red steps), and a proton (H^+) from either the solvent or a solvent exchangeable site in the active site of the enzyme (Scheme 1, blue steps).[1] Studies of pyranose 2-oxidase using rapid kinetics and substrate and solvent KIEs have shown that the transfer of the flavin-bound H, which originates from the α-carbon of the substrate and is transferred as a hydride ion (H^-) to the flavin N5 atom during substrate oxidation, is rate-determining for flavin oxidation.[12] Flavin reduction in choline oxidase also occurs through the transfer of a H^- from the α-carbon of the substrate, but the flavin is oxidized with the organic product of the reaction still present in the active site, as seen in Scheme 2. Indeed, it is the positive charge harbored on the reaction product that activates O_2 for reaction with the flavin rather than a protein charge as in the case of glucose oxidase.[17,18] If the wash-out of the H from the N5 atom of the reduced flavin is hampered in the presence of the reaction product, the opportunity exists to use substrate deuterium KIEs to report on the transfer of the H from the reduced flavin in O_2 reduction. Solvent deuterium KIEs, instead, will directly report on the H^+ transfer involving a solvent (exchangeable) site.

In this study, we have used pH effects, solvent effects, and KIEs, along with time-resolved absorbance spectroscopy of the oxidative half-reaction, to investigate the relative timing of H and H^+ transfers in the flavin oxidation reaction catalyzed by choline oxidase.

EXPERIMENTAL PROCEDURES

Materials. Choline chloride was bought from ICN. 1,2-[$^2H_4$]Choline bromide (98%) and sodium deuteroxide were purchased from Isostec Inc. (Miamisburg, OH). Deuterium oxide (99.9%) and deuterium chloride (99.5%) were obtained from Cambridge Isotope Co. (Andover, MA). Glycerol was from EMD. Recombinant choline oxidase from *Arthrobacter globiformis* strain ATCC 8010 was expressed from plasmid pET/*codA1* and purified

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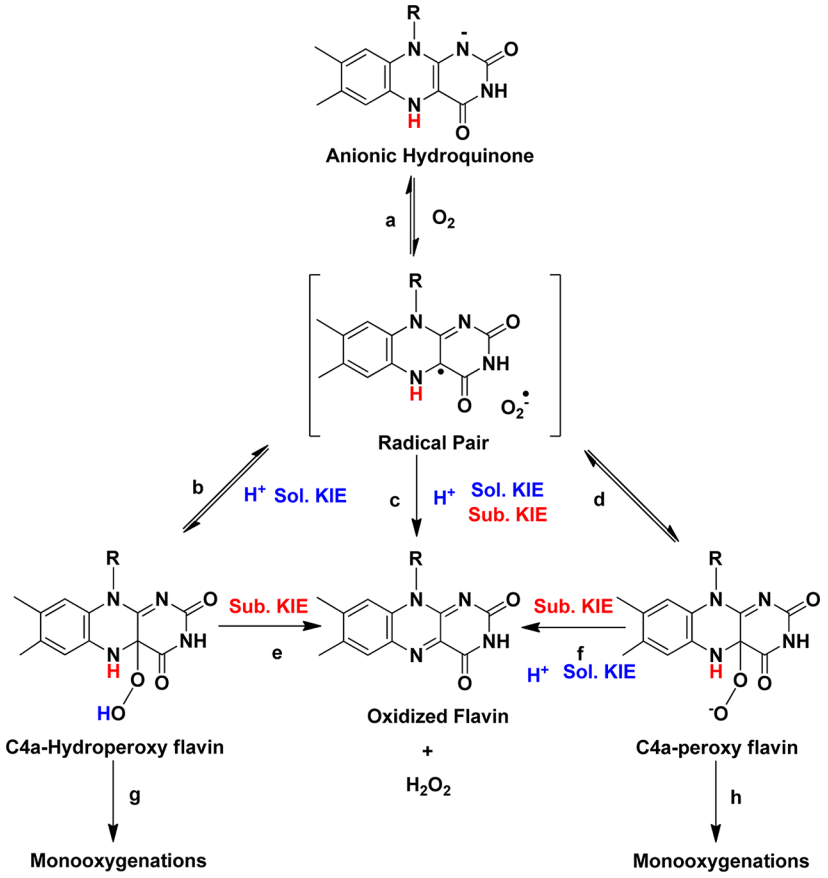
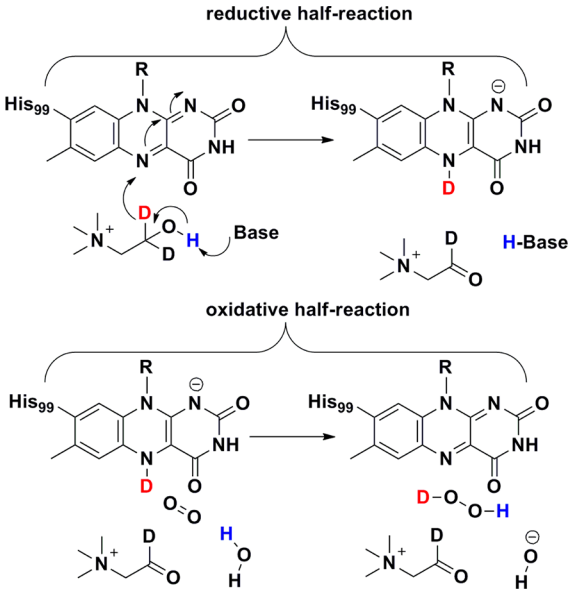
Scheme 1. Possible Routes for Flavin Oxidation in Flavoproteins



Scheme 2. Reductive and Oxidative Half-Reactions by Choline Oxidase with 1,2-[$^2H_4$]Choline as the Substrate[a]



[a]Note that the timings for the cleavages of the various bonds and the steps involved in O_2 activation are not shown here.

to homogeneity using a previously described protocol.[19] Completely oxidized enzyme was produced using a protocol described previously.[20] The kinetic parameters reported here have been normalized for flavin content per active site.

Kinetic Assays. The enzymatic activity was determined by measuring the rate of oxygen consumption polarographically using a computer-interfaced oxygen electrode from Hansatech. The steady-state kinetic parameters were determined using the method of initial rates with concentrations of choline or 1,2-[$^2H_4$]choline fixed at 25 mM and oxygen concentrations from 0.04 to 1.0 mM. As a starting step, the reaction mixture was equilibrated by bubbling in an O_2/N_2 gas mixture for approximately 15 min at the desired concentration. Enzymatic assays were initiated via the addition of 5 μL of choline oxidase at a final concentration of 0.12 μM to a final reaction volume of 1000 μL. Enzymatic assays were performed using sodium pyrophosphate or sodium phosphate in either H_2O or 99.9% D_2O at pL 8.0, 9.0, and 7.0. For buffers containing D_2O, the pD values were adjusted by adding 0.4 to the pH value.[21]

Solvent viscosity studies were conducted at pH 7.0, 8.0, and 9.0 using the procedure described above with glycerol as the viscosigen. On the basis of relative viscosities at 20 °C available from Lide,[22] the values at 25 °C were calculated.

Time-resolved absorbance spectroscopy in the double-mixing mode was performed using an SF-61DX2 Hi-Tech KinetAsyst high-performance stopped-flow spectrophotometer, thermostated at 25 °C, and equipped with a photo-diode-array detector. Choline oxidase was prepared fresh by gel filtration against 50 mM sodium phosphate (pD 7.0). The concentration of the enzyme before it was mixed with the substrate was 94 μM. 1,2-[$^2H_4$]Choline (130 μM) was prepared in 50 mM sodium phosphate (pD 7.0). The stopped-flow spectrophotometer was set up with a glucose/glucose oxidase scrubbing system at pH 6.0 the day before and left overnight. The buffer and substrate

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solutions contained in glass syringes were flushed with argon for approximately 1 h before being mounted on the stopped-flow spectrophotometer. The enzyme contained in a glass tonometer was subjected to 25 cycles of degassing by alternately flushing it with argon and applying vacuum. To ensure complete removal of the traces of oxygen, glucose (2 mM) and glucose oxidase (0.5 μM) were present in the anaerobic enzyme, buffer, and substrate solutions. Separately, glass syringes containing sodium phosphate buffer were saturated with 800, 540, 250, 140, and 40 μM O_2 before being mounted on the stopped-flow instrument. The aerobic syringes did not contain glucose or glucose oxidase. The first mixing in the stopped-flow spectrophotometer was between the anaerobic enzyme and anaerobic substrate, thereby producing reduced enzyme; this sample was allowed to age in the instrument until the enzyme-bound flavin had been completely reduced before being mixed with aerobic buffer at various oxygen concentrations. The time-resolved absorbance spectra were acquired 2.2 ms after the second mixing event.

Data Analysis. Data were fit using KaleidaGraph (Synergy Software, Reading, PA). The steady-state kinetic parameters at a fixed (25 or 50 mM) choline or 1,2-[$^2H_4$]choline concentration and varying O_2 concentrations were calculated using the Michaelis–Menten equation. Solvent viscosity effects on k_{cat}/K_{ox} (as a function of the concentration of O_2 as a substrate) (Figure 1B) were fit to eq 1

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

where $(k)_o$ and $(k)_\eta$ are the kinetic parameters in the absence and presence of glycerol as a viscosigen, respectively, S is the degree of dependence on viscosity, and η_{rel} represents the relative viscosity.

Individual traces at 456 nm for each oxygen concentration (Figure 2B) were fit to eq 2 using KinetAsyst 3 (TgK-Scientific, Bradford-on-Avon, U.K.)

$$A = -Be^{-\lambda t} + C \quad (2)$$

where A is the absorbance at the selected wavelength, B is the amplitude of the change in absorbance, C is the absorbance at infinite time, and λ is the observed first-order rate constant for flavin oxidation. The time-resolved absorbance spectra were analyzed with the global-fitting analysis software SPECFIT/32, with the best fit of the data obtained to an A → B kinetic model.

RESULTS AND DISCUSSION

To probe flavin oxidation during turnover of the enzyme, we used the $^{app}(k_{cat}/K_{ox})$ value determined at a fixed, saturating concentration of choline. Enzyme saturation with choline was experimentally demonstrated upon comparison of the $^{app}(k_{cat}/K_{ox})$ determined at pH 7.0 at two fixed substrate concentrations. With 25 and 50 mM choline, the $^{app}(k_{cat}/K_{ox})$ was essentially the same (120000 ± 5000 and 119000 ± 4000 M^{-1} s^{-1}, respectively). Similar results were obtained with 1,2-[$^2H_4$]choline (87000 ± 4000 and 87000 ± 5000 M^{-1} s^{-1}, respectively). These results agree well with published K_m values for choline (1.6 mM) and 1,2-[$^2H_4$]choline (2.5 mM) at pH 7.0, indicating that the enzyme is ≥90% saturated with 25 mM substrate.[23] Because previous kinetic investigations demonstrated a decrease in K_m with an increase in pH,[23] we conclude that the true k_{cat}/K_{ox} value, which is required to draw mechanistic conclusions, is approximated well at pH ≥7.0 by the $^{app}(k_{cat}/K_{ox})$ determined at 25 mM substrate.

Previous studies showed that the k_{cat}/K_{ox} with choline or 1,2-[$^2H_4$]choline as a substrate for choline oxidase is independent of pH between 6.0 and 10.0.[20,24] In principle, substitution of H_2O with D_2O might yield solvent effects on k_{cat}/K_{ox} due to changes in the isotopic composition of solvent exchangeable sites participating in the reaction (i.e., isotope effects), increases in the relative viscosity of the solution (i.e., viscosity effects), perturbations of observed pK_a values (i.e., pH effects), or a combination of these effects.[25−27] Mechanistic interpretation of solvent kinetic isotope effects requires that pH effects be ruled out and the contribution of relative viscosity to the measured effect be dissected. As illustrated in Figure 1A, the k_{cat}/K_{ox} with choline determined in D_2O was the same between pD 7.0 and 9.0, establishing a lack of pD effects. The normalized k_{cat}/K_{ox} at various concentrations of glycerol as a viscosigen increased with an inverse hyperbolic pattern with the increasing relative viscosity of the solvent (Figure 1B). While this pattern is consistent with an isomerization of the reduced enzyme in the oxidative half-reaction,[28] it also establishes that solvent KIEs are not inflated because of increased solvent viscosity of D_2O; indeed, they are slightly deflated. At 9% glycerol,[22] with a relative viscosity equivalent to that of D_2O (η = 1.25),[25] the inverse viscosity effect was ≤8% between pH 7.0 and 9.0. With these controls, solvent deuterium KIEs on k_{cat}/K_{ox} could be used to report on H^+ transfers involving solvent (exchangeable) sites, because there are no pH or pD effects and negligible, inverse viscosity effects.

When choline is substituted with 1,2-[$^2H_4$]choline as a substrate for choline oxidase, the enzyme-bound flavin acquires a D rather than an H on the N5 atom (Scheme 2).[24] A normal substrate deuterium KIE is expected on k_{cat}/K_{ox} in aqueous buffered solutions if the reaction of the reduced flavin with O_2 occurs prior to the potential wash-out of the D from the reduced flavin N5 atom to the solvent. Alternatively, both $^D(k_{cat}/K_{ox})_{H_2O}$ and $^D(k_{cat}/K_{ox})_{D_2O}$ should be equal to 1 if the N5 D atom of the reduced flavin is rapidly exchanged with the solvent. As illustrated in Table 1, significant substrate deuterium KIEs were determined in aqueous and deuterated buffered solutions at pL 7.0 and 8.0, consistent with negligible wash-out of the N5 H or D atom from the reduced flavin during enzyme turnover. Thus, substrate KIEs could be used to report on the H transfer from the reduced flavin during O_2 reduction.

With the enzyme in turnover at pL 7.0 or 8.0, the $^{D_2O}(k_{cat}/K_{ox})_H$ values were only slightly greater than 1 (Table 1). However, they increased when 1,2-[$^2H_4$]choline was used as a substrate instead of choline [$^{D_2O}(k_{cat}/K_{ox})_D$ > $^{D_2O}(k_{cat}/K_{ox})_H$ in Table 1], indicating that although small, the solvent KIE was significant. This is consistent with the transfer of the H^+ originating from the solvent, or a solvent exchangeable site, being manifested in the transition state for flavin oxidation catalyzed by the enzyme. At both pL values, the $^D(k_{cat}/K_{ox})_{H_2O}$ values were significantly greater than 1 (Table 1), and they increased further upon substitution of H_2O with D_2O [$^D(k_{cat}/K_{ox})_{D_2O}$ > $^D(k_{cat}/K_{ox})_{H_2O}$ in Table 1]. These data are consistent with transfer of the H from the flavin N5 atom also being manifested in the transition state for flavin oxidation. Multiple deuterium KIEs were consequently determined at pL 7.0 or 8.0 to elucidate whether the H and H^+ transfer reactions occurred in the same kinetic step or in different kinetic steps. $^{D,D_2O}(k_{cat}/K_{ox})$, which represents the effect of substituting choline in H_2O with 1,2-[$^2H_4$]choline in D_2O, was slightly larger than the product of the individual substrate and solvent KIEs [$^{D,D_2O}(k_{cat}/K_{ox})$ > $^D(k_{cat}/K_{ox})_{H_2O}$ × $^{D_2O}(k_{cat}/K_{ox})_H$ in Table 1]. These data rule out the possibility of H and H^+ transfers occurring in separate kinetic

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steps, for which multiple KIEs would be smaller than the product of the individual KIEs, and either substrate or solvent KIEs would have lower values when H_2O is substituted with D_2O or choline is substituted with 1,2-[$^2H_4$]choline, respectively.[29,30] Thus, a mechanism in which a C4a-hydroperoxy flavin is an intermediate in the oxidative pathway of the reaction (Scheme 1, route a-b-e), i.e., for which H and H^+ transfers would occur in separate kinetic steps, is ruled out.

Both the effects of isotopic substitution on the KIEs and the multiple KIEs are consistent with the transfers of H and H^+ from the flavin and the solvent (or a solvent exchangeable site) to O_2 occurring in the same kinetic step, in a synchronous[31] fashion. This kinetic step is partially rate-limiting in the oxidative half-reaction with the light isotopes (i.e., H and H^+) and is further slowed upon substitution of H with D or H^+ with D^+. This conclusion is supported by the increase in both the measured individual KIEs in the presence of the second isotopic substitution [$^{D_2O}(k_{cat}/K_{ox})_D > {}^{D_2O}(k_{cat}/K_{ox})_H$, and $^{D}(k_{cat}/K_{ox})_{D_2O} > {}^{D}(k_{cat}/K_{ox})_{H_2O}$] and the multiple KIE being larger than the product of the individual KIEs [$^{D,D_2O}(k_{cat}/K_{ox}) > {}^{D}(k_{cat}/K_{ox})_{H_2O} \times {}^{D_2O}(k_{cat}/K_{ox})_H$].[29] Mechanisms for flavin oxidation that bypass formation of a C4a-hydroperoxy flavin (Scheme 1, route a-c) or go through an anionic C4a-peroxy flavin intermediate (Scheme 1, route a-d-f), i.e., in which H and H^+ transfers occur in the same kinetic step, are thus consistent with the observed KIEs on the k_{cat}/K_{ox} values. In this regard, stabilization of an anionic C4a-peroxy flavin during oxidation of the flavin hydroquinone has been previously reported in cyclohexanone monooxygenase[11] and the oxygenase component of *p*-hydroxyphenylacetate 3-hydroxylase,[32] where the enzyme-bound flavin species have pK_a values of 8.4 and >10.0, respectively.[11,32] However, if an anionic C4a-peroxy flavin transiently forms in choline oxidase, its pK_a would need to be significantly less than 6, because no ionizable groups are seen in the pH profile of k_{cat}/K_{ox} with choline.[20]

If an anionic C4a-peroxy flavin were an obligatory intermediate during the oxidation of the hydroquinone in choline oxidase (Scheme 1, route a-d-f), it would transiently accumulate during the oxidation of the reduced flavin, allowing for its spectrophotometric detection. Stabilization of a C4a-(hydro)peroxy flavin has been recently reported in pyranose 2-oxidase.[12] To establish whether an anionic C4a-peroxy flavin is a reaction intermediate in the oxidative pathway, choline oxidase was mixed with a 1.4-fold molar excess of 1,2-[$^2H_4$]choline in a double-mixing stopped-flow spectrophotometer equipped with photodiode array detection, allowed to age until complete flavin reduction was achieved, and then mixed with various concentrations of O_2 in a deuterated buffered solution at pL 7.0 and 25 °C. Deuterated solvent and substrate, rather than choline and H_2O, were used to allow for a higher level of accumulation of the potential flavin intermediate in the oxidative half-reaction. Under these conditions, the oxidation of the enzyme-bound flavin proceeded without formation of any

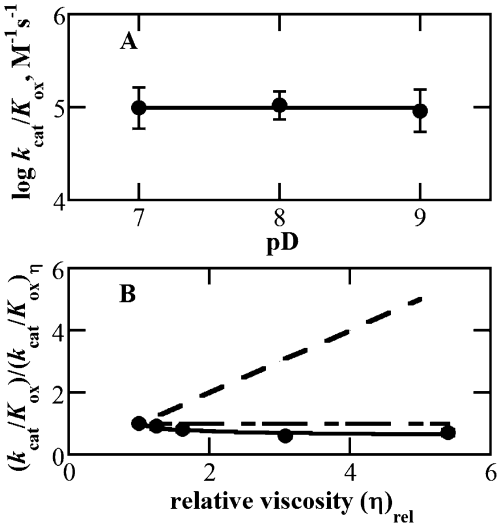

Figure 1. pD and viscosity contributions to solvent effects on k_{cat}/K_{ox}. (A) Log k_{cat}/K_{ox} for choline in D_2O as a function of pD. (B) Normalized k_{cat}/K_{ox} for choline at varying concentrations of glycerol as the viscosigen (0, 9, 18, 36, and 48%) as a function of relative viscosity at pH 8.0, with 25 mM choline and varying O_2 concentrations from 0.04 to 1 mM.

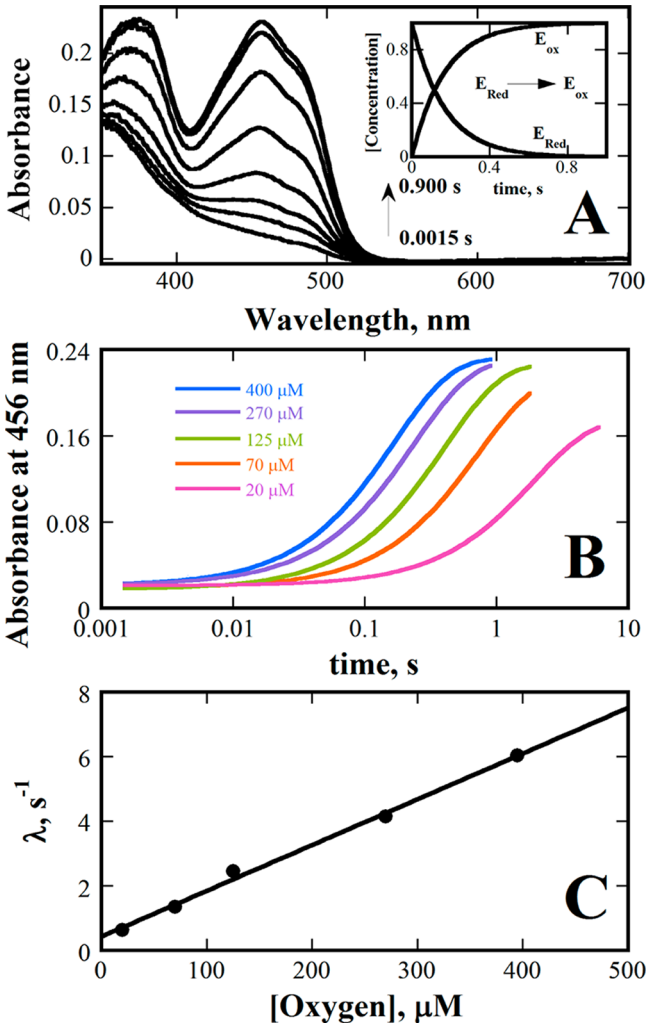

Figure 2. Time-resolved absorbance spectroscopy of the oxidation of reduced choline oxidase with O_2. The anaerobic enzyme was premixed with anaerobic 1,2-[$^2H_4$]choline (1.4-fold molar excess), allowed to age until complete flavin reduction had been achieved, and mixed with varying concentrations of O_2 in a double-mixing stopped-flow spectrophotometer equipped with PDA detection. Conditions: enzyme (24 μM after double mixing) in 50 mM sodium phosphate, pD 7.0, and 25 °C. All indicated times are after the end of flow, i.e., 2.2 ms. (A) Selected time-resolved absorbance spectra upon mixing reduced enzyme with 0.4 mM O_2. The inset shows multivariate analysis of the time-resolved spectra with an A → B kinetic model using SPECFIT/32, where E_{red} and E_{ox} are the reduced and oxidized enzymes, respectively. (B) Traces at 456 nm at varying O_2 concentrations. (C) Plot of the observed λ values as a function of O_2 concentration, which were fit to a linear function yielding $y = 0.014x + 0.4$ ($R^2 = 0.996$).

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Table 1. Deuterium Kinetic Isotope Effects on the Second-Order Rate Constant for Flavin Oxidation during Steady-State Turnover of Choline Oxidase

KIE	pL 7.0[a]	pL 8.0[b]
$^{D}(k_{cat}/K_{ox})_{H_2O}$	1.4 ± 0.1	1.6 ± 0.1
$^{D}(k_{cat}/K_{ox})_{D_2O}$	1.7 ± 0.1	2.1 ± 0.1
$^{D_2O}(k_{cat}/K_{ox})_{H}$	1.2 ± 0.1	1.09 ± 0.05
$^{D_2O}(k_{cat}/K_{ox})_{D}$	1.5 ± 0.1	1.4 ± 0.1
$^{D,D_2O}(k_{cat}/K_{ox})$	2.1 ± 0.4	2.2 ± 0.1
$^{D}(k_{cat}/K_{ox})_{H_2O} \times {}^{D_2O}(k_{cat}/K_{ox})_{H}$	1.7 ± 0.2	1.7 ± 0.1

[a]Experimental conditions: 50 mM sodium phosphate in H_2O or D_2O at 25 °C at 25 mM choline (H) or 1,2-[$^2H_4$]choline (D) and varying O_2 concentrations from 40 to 1000 μM. [b]Experimental conditions: 50 mM sodium pyrophosphate in H_2O or D_2O at 25 °C at 25 mM choline (H) or 1,2-[$^2H_4$]choline (D) and varying O_2 concentrations from 40 to 1000 μM.

intermediate (Figure 2A,B). In agreement with the lack of observable intermediates, a global fitting analysis of the time-resolved absorbance spectra yielded the best results with an A → B kinetic model (Figure 2A, inset). These results are not consistent with the chemical mechanism in which an anionic C4a-peroxyflavin is a reaction intermediate (Scheme 1, route a-d-f). Recent kinetic and computational studies of the oxidation of aryl-alcohol oxidase showed a lack of intermediates with the spectral properties of a C4a-intermediate, consistent with formation of such a species not being seen in that enzyme.[33]

The steady-state KIEs showed that the decay of the flavosemiquinone/$O_2^{-\bullet}$ radical pair is at least partially rate-limiting in the oxidative half-reaction. This suggests that a neutral flavosemiquinone intermediate should be observed when the reduced enzyme is reacted with O_2 (Scheme 1, route a-c). However, this is not the case (Figure 2). This apparent discrepancy can be explained with the effect of increasing solvent viscosity on the normalized k_{cat}/K_{ox} values (Figure 1B). Indeed, the inverse hyperbolic pattern to a limiting value of the normalized k_{cat}/K_{ox} with an increasing glycerol concentration demonstrates that the solvent effect does not originate from diffusion of O_2 to the reactive site, for which linear dependencies with slopes between 0 and 1 are expected, but rather with a slow isomerization of the reduced enzyme.[34] A slow isomerization of the reduced enzyme would mask subsequent kinetic events, i.e., formation and decay of the flavosemiquinone/$O_2^{-\bullet}$ radical pair, resulting in lack of detection of the flavosemiquinone and decreased values for the KIEs. The magnitude of the observed substrate KIE with values between 1.5 and 2.0, when compared to the expected effect of 3−4 for the cleavage of an N−H bond,[35] is consistent with the isotope sensitive kinetic step being significantly rate-limiting for the reaction of flavin oxidation. This suggests that, if formed, a C4a-peroxy flavin should accumulate to an extent that would allow its detection in rapid kinetics. Thus, the lack of detection of such a reaction intermediate suggests that its formation and decay are very unlikely in the reaction of choline oxidase.

The mechanism of flavin oxidation in choline oxidase directly results in the formation of oxidized flavin and hydrogen peroxide without stabilization of reaction intermediates, as evidenced from the rapid reaction kinetics and steady-state KIEs (Scheme 1, route a-c). This is in agreement with the accepted notion that flavoprotein oxidases do not stabilize reaction intermediates as demonstrated by using mechanistic probes with glucose oxidase,[16] and by using rapid kinetics with sarcosine oxidase,[36] *N*-methyltryptophan oxidase,[37] fructosamine oxidase,[38] vanillyl-alcohol oxidase,[39] monoamine oxidase (A and B),[40] D-amino acid oxidase,[41] and aryl-alcohol oxidase.[33] Notable exceptions are glycolate oxidase, for which an intermediate resembling a flavosemiquinone has been reported,[9] and pyranose 2-oxidase, for which a C4a-flavin intermediate has been observed.[12] The lack of stabilization of reaction intermediates, rather than their stabilization as for the case of flavoprotein monooxygenases, is likely a common feature of flavoprotein oxidases to minimize the potential leak of reactive oxygen species from the active site of the enzyme that are formed in the path from O_2 to H_2O_2.

In conclusion, pH effects, solvent viscosity effects, and KIEs, as well as time-resolved absorbance spectroscopy of the oxidative half-reaction, have been used in this study to elucidate the relative timing of H and H^+ transfers in the reaction of flavin oxidation catalyzed by choline oxidase. The results rule out a mechanism in which a C4a-hydroperoxy flavin forms in the oxidative pathway, for which H and H^+ transfers are not synchronous, while time-resolved absorbance spectroscopy showed the lack of detection of an anionic C4a-peroxy flavin. In contrast, the results of the mechanistic investigation are consistent with a mechanism for reduction of O_2 to H_2O_2 in which the H from the reduced flavin N5 atom and a H^+ from either the solvent or a solvent exchangeable site in the active site of the enzyme are transferred in the same kinetic step without any flavin-derived transient intermediates (Scheme 1, route a-c). This study represents the first instance in which the synchronous timing of H and H^+ transfers in the oxidation of a flavin was established in a flavoprotein oxidase. It complements previous studies of choline oxidase and other oxidases in which the mechanism for O_2 activation was probed with mechanistic tools.[4,16,17,36−38,42] This study also provides a framework for future studies of choline oxidase that will be aimed at the elucidation of the contribution of active site residues toward reduction of O_2.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Massey, V. (1994) Activation of molecular oxygen by flavins and flavoproteins. *J. Biol. Chem. 269*, 22459−22462.

(2) Mattevi, A. (2006) To be or not to be an oxidase: Challenging the oxygen reactivity of flavoenzymes. *Trends Biochem. Sci. 31*, 276−283.

(3) Halliwell, B., and Gutteridge, J. M. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J. 219*, 1−14.

(4) Klinman, J. P. (2007) How do enzymes activate oxygen without inactivating themselves? *Acc. Chem. Res. 40*, 325−333.

(5) Chaiyen, P., Fraaije, M. W., and Mattevi, A. (2012) The enigmatic reaction of flavins with oxygen. *Trends Biochem. Sci. 37*, 373−380.

(6) Eberlein, G., and Bruice, T. C. (1982) One and two electron reduction of oxygen by 1,5-dihydroflavins. *J. Am. Chem. Soc. 104*, 1449−1452.

(7) Ghisla, S., and Massey, V. (1989) Mechanisms of flavoprotein-catalyzed reactions. *Eur. J. Biochem. 181*, 1−17.

(8) Malmstrom, B. G. (1982) Enzymology of Oxygen. *Annu. Rev. Biochem. 51*, 21−59.
(9) Pennati, A., and Gadda, G. (2011) Stabilization of an intermediate in the oxidative half-reaction of human liver glycolate oxidase. *Biochemistry 50*, 1−3.
(10) Palfey, B. A., and McDonald, C. A. (2010) Control of catalysis in flavin-dependent monooxygenases. *Arch. Biochem. Biophys. 493*, 26−36.
(11) Sheng, D., Ballou, D. P., and Massey, V. (2001) Mechanistic studies of cyclohexanone monooxygenase: Chemical properties of intermediates involved in catalysis. *Biochemistry 40*, 11156−11167.
(12) Sucharitakul, J., Wongnate, T., and Chaiyen, P. (2011) Hydrogen peroxide elimination from C4a-hydroperoxyflavin in a flavoprotein oxidase occurs through a single proton transfer from flavin N5 to a peroxide leaving group. *J. Biol. Chem. 286*, 16900−16909.
(13) Thotsaporn, K., Chenprakhon, P., Sucharitakul, J., Mattevi, A., and Chaiyen, P. (2011) Stabilization of C4a-hydroperoxyflavin in a two-component flavin-dependent monooxygenase is achieved through interactions at flavin N5 and C4a atoms. *J. Biol. Chem. 286*, 28170−28180.
(14) Mallett, T. C., and Claiborne, A. (1998) Oxygen reactivity of an NADH oxidase C42S mutant: Evidence for a C(4a)-peroxyflavin intermediate and a rate-limiting conformational change. *Biochemistry 37*, 8790−8802.
(15) Roth, J. P., Wincek, R., Nodet, G., Edmondson, D. E., McIntire, W. S., and Klinman, J. P. (2004) Oxygen isotope effects on electron transfer to O_2 probed using chemically modified flavins bound to glucose oxidase. *J. Am. Chem. Soc. 126*, 15120−15131.
(16) Roth, J. P., and Klinman, J. P. (2003) Catalysis of electron transfer during activation of O_2 by the flavoprotein glucose oxidase. *Proc. Natl. Acad. Sci. U.S.A. 100*, 62−67.
(17) Gadda, G. (2012) Oxygen activation in flavoprotein oxidases: The importance of being positive. *Biochemistry 51*, 2662−2669.
(18) Gadda, G., Fan, F., and Hoang, J. V. (2006) On the contribution of the positively charged headgroup of choline to substrate binding and catalysis in the reaction catalyzed by choline oxidase. *Arch. Biochem. Biophys. 451*, 182−187.
(19) Fan, F., Ghanem, M., and Gadda, G. (2004) Cloning, sequence analysis, and purification of choline oxidase from *Arthrobacter globiformis*: A bacterial enzyme involved in osmotic stress tolerance. *Arch. Biochem. Biophys. 421*, 149−158.
(20) Ghanem, M., Fan, F., Francis, K., and Gadda, G. (2003) Spectroscopic and kinetic properties of recombinant choline oxidase from *Arthrobacter globiformis*. *Biochemistry 42*, 15179−15188.
(21) Schowen, K. B., and Schowen, R. L. (1982) in *Methods in Enzymology*, Vol. *87*, Academic Press, New York.
(22) Lide, D. R. (2000) *Handbook of Chemistry and Physics*, 81st ed., CRC Press, Boca Raton, FL.
(23) Fan, F., and Gadda, G. (2005) Oxygen- and temperature-dependent kinetic isotope effects in choline oxidase: Correlating reversible hydride transfer with environmentally enhanced tunneling. *J. Am. Chem. Soc. 127*, 17954−17961.
(24) Fan, F., and Gadda, G. (2005) On the catalytic mechanism of choline oxidase. *J. Am. Chem. Soc. 127*, 2067−2074.
(25) Karsten, W. E., Lai, C.-J., and Cook, P. F. (1995) Inverse Solvent Isotope Effects in the NAD-Malic Enzyme Reaction Are the Result of the Viscosity Difference between D_2O and H_2O: Implications for Solvent Isotope Effect Studies. *J. Am. Chem. Soc. 117*, 5914−5918.
(26) Quinn, D. M., and Sutton, L. D. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) pp 73−126, CRC Press, Boca Raton, FL.
(27) Schowen, K. B., and Schowen, R. L. (1982) in *Methods in Enzymology*, Vol. *87*, Academic Press, New York.
(28) Ansari, A., Jones, C., Henry, E., Hofrichter, J., and Eaton, W. (1992) The role of solvent viscosity in the dynamics of protein conformational changes. *Science 256*, 1796−1798.
(29) Cleland, W. W. (1982) Use of isotope effects to elucidate enzyme mechanisms. *CRC Crit. Rev. Biochem. 13*, 385−428.
(30) Edens, W. A., Urbauer, J. L., and Cleland, W. W. (1997) Determination of the Chemical Mechanism of Malic Enzyme by Isotope Effects. *Biochemistry 36*, 1141−1147.
(31) This study addresses the timing of H and H^+ transfers, but not whether they are mechanistically coupled. Thus, synchronous is preferred to concerted because it defines events that occur at the same time, rather than events that are performed in cooperation.
(32) Ruangchan, N., Tongsook, C., Sucharitakul, J., and Chaiyen, P. (2011) pH-dependent studies reveal an efficient hydroxylation mechanism of the oxygenase component of p-hydroxyphenylacetate 3-hydroxylase. *J. Biol. Chem. 286*, 223−233.
(33) Hernández-Ortega, A., Lucas, F., Ferreira, P., Medina, M., Guallar, V., and Martínez, A. T. (2012) Role of Active Site Histidines in the Two Half-Reactions of the Aryl-Alcohol Oxidase Catalytic Cycle. *Biochemistry 51*, 6595−6608.
(34) Yuan, H., Xin, Y., Hamelberg, D., and Gadda, G. (2011) Insights on the Mechanism of Amine Oxidation Catalyzed by D-Arginine Dehydrogenase through pH and Kinetic Isotope Effects. *J. Am. Chem. Soc. 133*, 18957−18965.
(35) Jencks, W. P., and Carriuolo, J. (1961) General Base Catalysis of Ester Hydrolysis. *J. Am. Chem. Soc. 83*, 1743−1750.
(36) Zhao, G., Bruckner, R. C., and Jorns, M. S. (2008) Identification of the oxygen activation site in monomeric sarcosine oxidase: Role of Lys265 in catalysis. *Biochemistry 47*, 9124−9135.
(37) Bruckner, R. C., Winans, J., and Jorns, M. S. (2011) Pleiotropic impact of a single lysine mutation on biosynthesis of and catalysis by N-methyltryptophan oxidase. *Biochemistry 50*, 4949−4962.
(38) McDonald, C. A., Fagan, R. L., Collard, F., Monnier, V. M., and Palfey, B. A. (2011) Oxygen reactivity in flavoenzymes: Context matters. *J. Am. Chem. Soc. 133*, 16809−16811.
(39) Fraaije, M. W., and van Berkel, W. J. (1997) Catalytic mechanism of the oxidative demethylation of 4-(methoxymethyl)phenol by vanillyl-alcohol oxidase. Evidence for formation of a p-quinone methide intermediate. *J. Biol. Chem. 272*, 18111−18116.
(40) Tan, A. K., and Ramsay, R. R. (1993) Substrate-specific enhancement of the oxidative half-reaction of monoamine oxidase. *Biochemistry 32*, 2137−2143.
(41) Rosini, E., Molla, G., Ghisla, S., and Pollegioni, L. (2011) On the reaction of D-amino acid oxidase with dioxygen: O_2 diffusion pathways and enhancement of reactivity. *FEBS J. 278*, 482−492.
(42) Roth, J. P. (2009) Oxygen Isotope Effects as Probes of Electron Transfer Mechanisms and Structures of Activated O_2. *Acc. Chem. Res. 42*, 399−408.