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Stabilization of an Intermediate in the Oxidative Half-Reaction of Human Liver Glycolate Oxidase†

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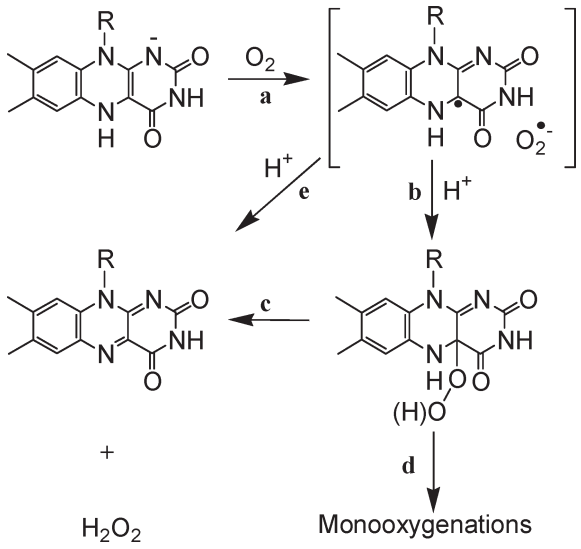
ABSTRACT: Glycolate oxidase is a flavin-dependent enzyme that catalyzes the oxidation of α-hydroxy acids to the corresponding α-keto acids, with reduction of molecular oxygen to hydrogen peroxide. A number of probes have been used to investigate the oxidative half-reaction catalyzed by the enzyme, including steady state and rapid kinetics, pH studies, solvent kinetic isotope effects, and solvent viscosity effects. Here we present the first spectroscopic evidence of the formation of an intermediate with absorbance features resembling those of a flavosemiquinone in the oxidative half-reaction of glycolate oxidase.

The flavin, i.e., 7,8-dimethylisoalloxazine, is the most common cofactor found in oxidases and monooxygenases. In fact, the reduced flavin is one of the few biocatalysts, along with copper and iron, that can effectively reduce molecular oxygen to hydrogen peroxide or water, in reactions that are controlled and modulated by the protein microenvironment surrounding the flavin (*1*, *2*). The initial step in the oxidation of the flavin has been proposed on the basis of chemical reasoning to be the transfer of a single electron from the hydroquinone, i.e., the two-electron reduced flavin, to molecular oxygen to yield the radical pair superoxide anion and flavin semiquinone (**a** in Scheme 1) (*1*−*5*). The radical pair can then collapse on the C4a atom of the flavin to form a (hydro)peroxyflavin (**b**) (*1*, *2*), which would subsequently generate hydrogen peroxide and oxidized flavin (**c**) or hydroxylate organic molecules (**d**). Alternatively, a second electron can be transferred directly from the flavin semiquinone to the superoxide anion, bypassing the formation of a C4a-flavin adduct (**e**). The stabilization of the C4a (hydro)peroxyflavin is a common feature that is unequivocally established in the flavin-dependent monooxygenases (*6*, *7*). In contrast, the oxidative pathway followed by flavin-dependent oxidases is still a matter of debate, because most of the attempts to detect early intermediates have failed (*2*, *8*, *9*). The crystal structures of several flavoprotein oxidases support the direct formation of hydrogen peroxide because in the active site there is not enough space to accommodate a C4a intermediate (*2*, *8*, *9*). Nonetheless, a C4a-flavin adduct with oxygen was recently trapped in the crystal structure of the *Arthrobacter globiformis* choline oxidase, demonstrating that the intermediate can be accommodated in the active site of this enzyme (*10*). Furthermore, spectroscopic evidence of the transient formation of a C4a-hydroperoxyflavin in oxidases has been reported for pyranose 2-oxidase from *Trametes multicolor* (*9*, *11*).

Scheme 1



In no case, however, has the proposed flavin semiquinone been observed in the oxidation of a flavin-dependent oxidase, most likely because its rate of decay is greater than the rate of formation.

In this work, we have used a number of probes to investigate the oxidative half-reaction of a representative flavoprotein oxidase, namely human liver glycolate oxidase. Using time-resolved spectroscopic studies under conditions in which later kinetic steps are decelerated (either **b** or **e**), we present the direct observation of a flavin intermediate with absorbance features resembling those of a flavosemiquinone in the oxidative half-reaction of a flavin-dependent oxidase.

When reduced glycolate oxidase is mixed with oxygenated buffer in a stopped-flow spectrophotometer in aqueous buffered solutions adjusted between pH 5.0 and 10.0, the enzyme-bound flavin is oxidized in a monophasic pattern without accumulation of any detectable reaction intermediate. Accordingly, the rapid kinetic traces at 390 or 450 nm were fit best to a single-exponential process (eq 1 of the Supporting Information). The observed rate constants acquired at varying O_2 concentrations were linearly dependent on the O_2 concentration (eq 3 of the Supporting Information), allowing for the determination of the second-order rate constant for the oxidative half-reaction and its reverse (k_{ox} and k_{rev} in Scheme S1 of the Supporting Information). The k_{ox} value increases with increasing pH between 8000 ± 1000 M^{-1} s^{-1} at low pH and 44000 ± 1000 M^{-1} s^{-1} at high pH, yielding a pK_a of 6.8 ± 0.2 (Figure S1 of the Supporting Information). The k_{rev} is pH-independent, with an average value

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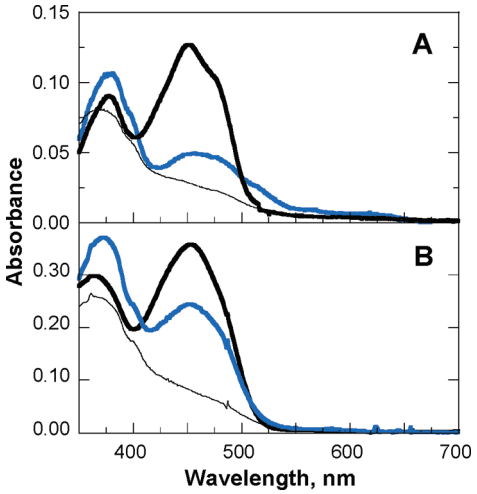

FIGURE 1: (A) Oxidative half-reaction of glycolate oxidase in 100 mM piperazine (pD 5.0) at 30 °C: reduced enzyme (thin black line), oxidized enzyme (thick black line), and intermediate I recorded 2.2 ms after mixing (blue line). (B) Anaerobic reduction of glycolate oxidase mediated by xanthine/xanthine oxidase in 100 mM NaH_2PO_4 (pH 7.0): oxidized enzyme (thick black line), reduced enzyme (thin black line), and anionic red semiquinone (blue line).

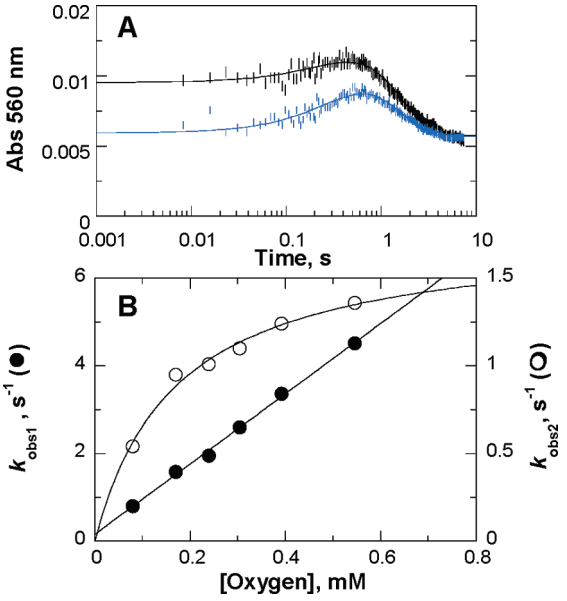

FIGURE 2: Oxidation of reduced glycolate oxidase with O_2 in 100 mM piperazine (pD 5.0) at 30 °C. (A) The time courses of the reactions are shown at 560 nm, with 0.17 (blue) and 0.55 mM O_2 (black). (B) Observed rate constants for the fast and slow phases of flavin oxidation are plotted vs O_2 concentration, with the data fit with eqs 4 and 5 of the Supporting Information.

of 0.8 ± 0.3 s^{-1} (Table S1 of the Supporting Information). This pH pattern is indistinguishable from the pH profile of the second-order rate constants obtained by using steady state kinetics recently reported for the enzyme (Figure S1 of the Supporting Information) (*12*).

The UV−visible absorbance spectrum of the reduced enzyme exhibited a well-defined maximum at 360 nm throughout the pH range considered irrespective of the solvent used, as shown for pD 5.0 in Figure 1A. Thus, the ionization of the N1 atom of the reduced flavin, which in solution has a pK_a of 6.7 (*13*, *14*), cannot account for the 10-fold difference in the k_{ox} values determined at low and high pH by using steady state and rapid reaction techniques. Instead, the pK_a of 6.8 may reflect a change in the rate-determining step that modulates the reactivity of the reduced flavin with oxygen (*12*).

In the mechanism of Scheme 1, the postulated radical pair composed of the flavin semiquinone and superoxide anion decays through proton-sensitive kinetic steps, irrespective of whether a C4a-hydroperoxyflavin is formed (**b**) or not formed (**e**). Consequently, solvent kinetic isotope effects were used to probe whether the proton-sensitive kinetic step associated with the decay of the postulated radical pair was (at least) partially rate-limiting for flavin oxidation at either low or high pH. The $^{D_2O}(k_{cat}/K_{oxygen})$ values were determined by measuring initial rates at varying concentrations of O_2 and saturating glycolate concentrations 2 pH units below and above the pK_a of 6.8. At pL 5.0, the solvent isotope effect was 1.4 ± 0.2, whereas at pL 9.0, it was not different from 1.0 (Figure S2 of the Supporting Information). The solvent effects determined at low pH could originate from proton transfers or from the diffusion of O_2 to the reaction site, which could be affected in D_2O because of the increased solvent viscosity with respect to aqueous buffered solutions (*15*). This was probed by determining the steady state kinetic parameters in the presence of increasing concentrations of glycerol (between 9 and 48%). Plots of the normalized k_{cat}/K_{oxygen} values as a function of the relative viscosity of the buffered solutions yielded lines with negligible slopes of 0.02 ± 0.06 at pH 5.0 and 0.07 ± 0.06 at pH 9.0 (Figure S3 of the Supporting Information). These data established that no solvent viscosity-sensitive steps limit the k_{cat}/K_{oxygen}-determining step for the oxidative half-reaction.

The combination of pH, solvent kinetic isotope effects, and solvent viscosity effects in the steady state kinetic approach (vide ante) establishes that a proton transfer is at least partially rate-limiting for the oxidative half-reaction of glycolate oxidase when D_2O substitutes for water at low pH. On the basis of the minimal mechanism of Scheme 1, we hypothesized that in D_2O at pD 5.0 the postulated transient flavin semiquinone could be observed because of subsequent proton transfer reaction(s) being slower than that in water. A stopped-flow spectrophotometer equipped with a photodiode array detector was employed to acquire time-resolved absorbance spectra between 350 and 700 nm of the enzyme while it was reacting with O_2 in D_2O at pD 5.0 (Figure 1 and Figure S4A of the Supporting Information). As shown in Figure 2A, the reduced enzyme was oxidized in a biphasic pattern, immediately establishing the formation of a transient intermediate in the oxidation of the reduced flavin with peaks centered at 377 and 450 nm and a broad absorbance band around 530−600 nm. Traces at 560 nm displayed initial increases in absorbance before decreasing at longer times (Figure 2A). Similar patterns were observed at 390 nm (Figure S4B of the Supporting Information). In contrast, the traces at 450 nm showed exclusively increases in absorbance (Figure S4C of the Supporting Information). A fit of the kinetic traces to a double-exponential process (eq 2 of the Supporting Information) allowed for the determination of the rate constants (k_{obs}) for the fast and slow phases at various O_2 concentrations. A plot of the k_{obs} values for the fast phase as a function of O_2 concentration yielded a straight line (Figure 2B), allowing the determination of a second-order rate constant for the reaction of the reduced flavin with oxygen (k_1 in Scheme 2) of 5600 ± 300 M^{-1} s^{-1} and a first-order rate constant for the reverse reaction (k_2) of 0.20 ± 0.05 s^{-1}. A plot of the k_{obs} values for the slow phase as a function of O_2 concentration yielded a hyperbola with a zero intercept (Figure 2B),

Scheme 2: Oxidation of Reduced Glycolate Oxidase in a Deuterium Oxide-Buffered Solution at pD 5.0

$$\text{Ered} + O_2 \underset{k_2\ 0.2\ s^{-1}}{\overset{k_1\ 5{,}600\ M^{-1}s^{-1}}{\rightleftharpoons}} \text{Intermediate I} \xrightarrow{k_3\ 1.5\ s^{-1}} \text{Eox} + H_2O_2$$

allowing the determination of a first-order rate constant for the decay of intermediate I to oxidized flavin (k_3) of 1.5 s^{-1} and establishing a negligible rate for the reverse reaction. With saturating glycolate (10 mM) and 0.6 mM O_2, the apparent turnover rate of the enzyme at pD 5.0 was 1.2 s^{-1}. This value compares well to the k_{obs2} value of 1.4 s^{-1} measured in the stopped flow under similar conditions, establishing the decay of intermediate I in the catalytic pathway of the enzyme. A control experiment in which the oxidation of the reduced enzyme was studied in a stopped-flow spectrophotometer at pD 9.0 yielded a monophasic kinetic behavior similar to those observed in aqueous buffered solutions (Figure S5 of the Supporting Information). Thus, intermediate I is observed only below the pK_a value of 6.8 when solvent-sensitive kinetic step(s) are slowed in D_2O.

As shown by the blue trace in Figure 2A, at a high concentration of oxygen (i.e., 0.55 mM), intermediate I is already being formed within the dead time of the stopped-flow spectrophotometer (2.2 ms). Consistent with this observation, the absorbance spectrum of glycolate oxidase recorded immediately after mixing of the reduced enzyme with 0.55 mM oxygen at pD 5.0 does not show the typical features of the hydroquinone, which in glycolate oxidase presents a well-resolved peak at 360 nm and no significant absorbance above 550 nm (Figure 1). In contrast, well-resolved maxima are centered at 377 and 455 nm, with a 377 nm:455 nm ratio of 2.2, and absorbance at 525−650 nm (Figure 1). These spectroscopic features may suggest the presence of a flavin semiquinone and not a C4a-flavin adduct, because the latter is devoid of absorbance above 500 nm (*15*). In this regard, the UV−visible spectrum of intermediate I with well-resolved maxima at 377 and 455 nm and a defined shoulder at 400 nm is similar to that of an anionic red flavin semiquinone obtained during the anaerobic reduction of glycolate oxidase mediated by xanthine/xanthine oxidase (Figure 1 and Figure S6 of the Supporting Information). Differences may be due to the presence of the neutral semiquinone in intermediate I, which is not stabilized in the anaerobic reduction mediated by xanthine/xanthine oxidase. Rapid-freeze quench EPR will be used to unequivocally establish whether intermediate I is a flavin semiquinone and its identity, i.e., anionic red, neutral red, or neutral blue (*16*).

In summary, we have used a number of mechanistic probes and time-resolved spectroscopy to investigate the oxidation of the reduced flavin in glycolate oxidase. Under conditions in which later proton transfer steps are slowed by using deuterium oxide, we have presented evidence of the observation of a transient species with absorbance features of a flavosemiquinone in the oxidation of a flavin-dependent oxidase. Future studies will address the nature of intermediate I in the reaction of flavin oxidation catalyzed by glycolate oxidase.

SUPPORTING INFORMATION AVAILABLE

Supplementary figures and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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