The Reaction of Reduced Xanthine Dehydrogenase with Molecular Oxygen

REACTION KINETICS AND MEASUREMENT OF SUPEROXIDE RADICAL*

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Xanthine dehydrogenase (XDH) from bovine milk contains significant activity in xanthine/oxygen turnover assays. The oxidative half-reaction of XDH with molecular oxygen has been studied in detail, at 25 °C, pH 7.5, to determine the basis of the preference of XDH for NAD over oxygen as oxidizing substrate. Spectral changes of XDH accompanying oxidation were followed by stopped-flow spectrophotometry. The amount of superoxide radicals formed during oxidation was investigated to assess the ability of XDH to catalyze production of oxygen radicals. Reduced XDH reacts with oxygen in at least 4 bi-molecular steps, with 1.7–1.9 mol of superoxide per mol of XDH formed from the last 2 electrons oxidized. A model is discussed in which the flavin hydroquinone transfers electrons to oxygen to produce hydrogen peroxide at a rate constant of at least 72,000 s⁻¹, whereas flavin semiquinone reduces oxygen to form superoxide as slow as 16 s⁻¹.

Steady-state kinetics of xanthine/oxygen and NADH/oxygen turnover of XDH were determined to have $k_{cat}$ values of 2.1 ± 0.1 and 2.5 ± 0.9 s⁻¹, respectively, at 25 °C, pH 7.5. XDH is therefore capable of catalyzing the formation of reduced oxygen species at one-third the rate of xanthine/NAD turnover, 63 s⁻¹ (Hunt, J., and Massey, V. (1992) J. Biol. Chem. 267, 21479–21485), in the absence of NAD. As XDH contains a significant and intrinsic xanthine oxidase activity, estimates of relative amounts of XO and XDH based solely upon turnover assays must be made with caution. Initial-rate assays containing varying amounts of xanthine, NAD, and oxygen indicate that at 100% oxygen saturation, NADH formation is only inhibited at concentrations of xanthine and NAD below $K_m$ for each substrate.

Purine catabolism in primates ends with the xanthine oxidoreductase-catalyzed oxidation of xanthine to urate with concomitant reduction of either molecular oxygen or NAD. This enzyme can be isolated from bovine milk in two different forms, xanthine oxidase (XO) and xanthine dehydrogenase (XDH). The enzyme type depends on the oxidation state of protein thiols, and the two forms are interconvertible (1, 2). Preincubation with thiol-reducing agents produces XDH, which contains high dehydrogenase activity and low oxidase activity. When the enzyme is isolated from milk without precaution to prevent disulfide formation, it is obtained as XO form and has high oxidase activity and very little dehydrogenase activity. Xanthine oxidoreductase represents a unique system in which two fundamentally different types of reactions, electron transfer to NAD or to molecular oxygen, can be catalyzed by the same protein, differing only as a result of a conformational change.

Xanthine oxidoreductase exists as a homodimer of 145-kDa subunits. Each subunit contains one FAD, one molybdopterin, and two 2Fe/2S clusters of the spinach ferredoxin type (3, 4). Reducing substrates of the purine type, such as xanthine, react at the molybdenopterin site of the enzyme (5). Oxidizing substrates, such as NAD or oxygen, react at the flavin center (6, 7). These cofactors can accept a total of six electrons from 3 mol of xanthine. When reduced by artificial electron donors, an additional pair of electrons can be reversibly accepted by a cystine which is not involved in the XO/XDH interconversion (8). Both the 2Fe/2S and FAD moieties undergo distinct spectral changes on reduction, providing a sensitive means for monitoring the redox state of the enzyme.

Xanthine oxidoreductase catalysis can be separated into a reductive half-reaction in which 2 electrons at a time are transferred from xanthine to the enzyme and an oxidative half-reaction in which electrons are conveyed from the enzyme to oxygen or NAD. The reaction of reduced XDH with oxygen was investigated to determine the basis for the predisposition of XDH toward NAD, instead of oxygen, as an oxidizing substrate. XDH is known to contain an NAD binding site (1), adjacent to the flavin moiety, which is absent in XO. Also, the flavin midpoint potential of XDH, −340 mV, is sufficiently low for reduction of NAD, −335 mV at pH 7.5 (9). In contrast, the flavin midpoint potential of XO, −255 mV (10), is too high to significantly reduce NAD. These properties demonstrate that XDH, and not XO, is equipped to react with NAD. The current study addresses how these changes influence oxidation by oxygen, with particular regard to rate and products of reaction. Rates of reaction were measured by mixing pre-reduced XDH with oxygen in a stopped-flow spectrophotometer, monitoring the oxidation of iron-sulfur and flavin centers. Reduction of O₂ by reduced flavins can result in the formation of H₂O₂ or O₂⁻. These activated oxygen species are thought to be the agents of oxidative stress acting in ischemia and the aging process. Superoxide produced was quantitated by superoxide-dependent reduction of cyt c.

The oxidative half-reaction with oxygen has been well-studied for two closely related enzymes, bovine milk XO and chicken liver XDH. In the case of XO, oxidation is triphasic.

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The abbreviations used are: XO, xanthine oxidase; XDH, xanthine dehydrogenase; XDHₙ₋₋, xanthine dehydrogenase reduced by n electrons; cyt c, cytochrome c; SOD, superoxide dismutase; MMTS, methylimethane thiosulfonate.

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with the first phase a lag, the second phase saturating at a rate of 125 s\(^{-1}\), and the third phase showing bi-molecular kinetics at 10,000 m\(^{-1}\) s\(^{-1}\), at 25 °C, pH 8.5 (11–13). Product analysis yielded 2 mol of O\(_2\) and 2 mol of H\(_2\)O per mol of XO, with the O\(_2\) produced from the last 2 electrons to leave XO. In the case of chicken liver XDH, four reaction phases were measured with the first two having rate constants of 1,900 m\(^{-1}\) s\(^{-1}\) and 260 m\(^{-1}\) s\(^{-1}\), and the last two being a complex combination of rates (14). These experiments were done at 4 °C, pH 7.8. With chicken liver XDH, 3 mol of O\(_2\) and 1.5 mol of H\(_2\)O were produced on oxidation. Superoxide was produced from the last electrons to leave chicken liver XDH as well.

In the current study with the mammalian bovine milk XDH, the oxidative half-reaction with oxygen proceeds via at least 4 bi-molecular reactions with observed rate constants of 72,000 ± 16,000, 3,500 ± 1,300, 120 ± 21, and 16 ± 3.5 m\(^{-1}\) s\(^{-1}\). From 1.7–1.9 mol of O\(_2\) per mol of XDH is formed from the last 2 electrons oxidized. A mechanism is proposed in which FADH\(_2\) reacts with oxygen to form H\(_2\)O\(_2\) and FAD at a microscopic rate constant of 1,700 m\(^{-1}\) s\(^{-1}\), while FADH\(_2\) reacts with oxygen to form O\(_2\) and FAD as slow as 16 m\(^{-1}\) s\(^{-1}\). Observed rate constants are thought to progressively decrease with the fraction of the relevant reduced flavin species, FADH\(_2\) or FADH, as well as with the increasing redox potential of XDH.

In addition, the competence of XDH to catalyze xanthine/oxygen and NADH/oxygen turnover was determined by measuring the steady-state kinetics of these two activities by the method of initial rates. Both activities gave parallel or near-parallel lines on Lineweaver-Burk plots, consistent with ping-pong mechanisms. Xanthine/oxygen and NADH/oxygen turnover have \(k_{cat}\) values of 2.1 ± 0.1 and 2.5 ± 0.9 s\(^{-1}\), respectively, at 25 °C, pH 7.5. These rates are 33% and 40% that of xanthine/NAD turnover, \(k_{cat}\) of 6.3 s\(^{-1}\) (1), indicating that XDH-catalyzed reduction of oxygen in vitro is significant in the absence of sufficient competing NAD.

The ability of oxygen to compete with NAD as a substrate was determined by measuring the inhibition of xanthine/NAD turnover as a function of oxygen concentration. Significant inhibition of NADH formation under initial-rate conditions was only observed at 100% oxygen saturation (1.2 mM at 25 °C) and at concentrations of xanthine and NAD near their \(K_m\) values, ≤1 and 7 \(\mu\)M (1), respectively. Oxygen competes very poorly with NAD as an alternative substrate for XDH. By monitoring the entire reaction with an oxygen electrode, however, oxygen consumption occurs slowly, even in the presence of 1 mM NAD. Once the xanthine/NAD reaction has gone to completion, the NADH produced can be consumed by the NADH oxidase activity of XDH.

**Materials and Methods**

Xanthine dehydrogenase was purified by the method of Hunt and Massey (1). Concentrations expressed are per monomer, determined with an extinction coefficient at 450 nm of 37,800 m\(^{-1}\) cm\(^{-1}\). The percent functional enzyme was measured prior to each usage by determining the fraction of absorbance lost at 295 nm on anaerobic reduction by 200 \(\mu\)M xanthine, with extinction increases of 7,000 per 2Fe/2S center, 12,200 for XDH 2e, 14,500 for XDH 5e, in units of \(M\) cm\(^{-1}\), and 29,600 for XDH 1e, in units of \(M\) cm\(^{-1}\). The percent functional enzyme was measured prior to each usage by determining the fraction of absorbance lost at 295 nm on anaerobic reduction by 200 \(\mu\)M xanthine, with extinction increases of 7,000 per 2Fe/2S center, 12,200 for XDH 2e, 14,500 for XDH 5e, in units of \(M\) cm\(^{-1}\), and 29,600 for XDH 1e, in units of \(M\) cm\(^{-1}\).

**Preparation of Desulfo XDH**—The very closely related xanthine oxidase is known to be inactivated by cyanolysis of the labile molybdenum site sulfur (16). Desulfo XDH was prepared by incubating a 200 mM solution of XDH with 35 mM potassium cyanide at 25 °C, pH 7.5, until no residual activity remained. The sample was passed over a Sephadex G-25 column to remove excess potassium cyanide. Inactive XDH was then prepared for an oxidative half-reaction experiment as described above.

**Superoxide Detection**—Superoxide formed in the reaction of reduced XDH with oxygen was measured as the superoxide dismutase (SOD) inhibited reduction of cytochrome c (cyt c) (17). Samples containing 6.5 \(\mu\)M XDH, 20 mM potassium oxalate, 0.65 \(\mu\)M 5-Deazaflavin, 10 \(\mu\)g/ml catalase, 500 \(\mu\)M methylmethane thiosulfonate (MMTS), and 10 \(\mu\)g/ml SOD (when used) in a total volume of 1 ml were held at 25 °C for 10 min. MMTS was included to derivatize cysteines on XDH. This modification inhibited reduction of cyt c which was observed on mixing oxidized XDH with cyt c. Following preincubation, samples of XDH were made anaerobic in an anaerobic cuvette and then photo-reduced. The cuvette was then opened to air, and 25 \(\mu\)l of 2 mM cyt c was added (50 \(\mu\)M final), followed by immediate mixing with air. Reduction of cyt c was followed...
served rates of 1.4 and 0.04 min$^{-1}$ were produced. The reaction was initiated by tipping in NAD from hydrogenase. A strong thermodynamic driving force is supplied (see "Materials and Methods").

Approximately 7 nmol of reduced XDH was reacted with the NADH-generating system was reduced at E$$_5$$550). The number of remaining electrons in each intermediate was calculated from the activity of XO and XDH toward NADH was used. A sample of 3.8 mM XDH was reacted anaerobically with an NADH-generating system containing 1 mM NAD, 1 mM glucose 6-phosphate, and 5 units/ml of Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase. A strong thermodynamic driving force is supplied by spontaneous hydrolysis of the glucose-6-phosphate 1,5-lactone produced. The reaction was initiated by tipping in NAD from the side arm of an anaerobic cuvette. The XDH sample was reduced bihapically by the NADH-generating system at observed rates of 1.4 and 0.04 min$^{-1}$. At the end of 3 h, 95% of the absorbance at 450 nm was bleached, relative to reduction with excess sodium dithionite (not shown). In contrast, 3.8 µM XO reacted with the NADH-generating system was reduced at approximately 7 x 10$^{-4}$ min$^{-1}$. This reaction was not followed to completion; however, at 26 h the XO sample was only 61% reduced (not shown). As XO is reduced by NADH much more slowly than XDH, these experiments are consistent with the conclusion that the 95% bleaching of the XDH sample is due to reduction of the dehydrogenase form enzyme and that the oxidase form constitutes no more than 5% of the total sample.

**Spectral Intermediates in the Reaction with Oxygen**—To determine the number and type of species in the reaction with oxygen, fully reduced XDH was reacted with oxygen in the diode array stopped-flow instrument. It should be noted that only the iron-sulfur and FAD centers undergo significant changes in the visible spectrum; oxidation of the molybdenum center is not directly observed in these experiments. A solution of dithionite-reduced XDH (17 µM after mixing) was reacted with 610 µM oxygen at 25 °C, pH 7.5. Spectra were recorded from 1.25 ms to 30 min, with a logarithmic bias to the distribution of data points. Spectra were analyzed with the Specfit program from 400 to 650 nm, as inclusion of the entire data set contained more data points than the analysis program could accommodate. Evolving factor analysis of the data by singular value decomposition indicated that four species were present in the reaction, with the possibility of up to two additional species. Spectra were fit to a five-species consecutive model due to the observation of four reaction phases in single-wavelength stopped-flow data (see below). Spectra of enzyme species derived from a four-exponential free fit to the diode array data are shown in Fig. 1. Observed rates from this free fit are $k_1$ = 22 s$^{-1}$, $k_2$ = 2.5 s$^{-1}$, $k_3$ = 0.15 s$^{-1}$, and $k_4$ = 0.011 s$^{-1}$. To determine the oxidation state of each reaction species, spectra were analyzed by comparing the extinction at 450 nm to that calculated for XDH at discrete reduction states (see "Materials and Methods"). From this analysis (Table I) it is clear that

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### RESULTS

**Quantitation of Fraction of Xanthine Oxidase**—The amount of residual XO-type enzyme in preparations of XDH was measured to distinguish the inherent reactivity of XDH toward oxygen from that of contaminating XO. The difference in reactivity of XO and XDH toward NADH was used. A sample of 3.8 µM XDH was reacted anaerobically with an NADH-generating system containing 1 µM NAD, 1 mM glucose 6-phosphate, and 5 units/ml of Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase. A strong thermodynamic driving force is supplied by spontaneous hydrolysis of the glucose-6-phosphate 1,5-lactone produced. The reaction was initiated by tipping in NAD from the side arm of an anaerobic cuvette. The XDH sample was reduced bihapically by the NADH-generating system at observed rates of 1.4 and 0.04 min$^{-1}$. At the end of 3 h, 95% of the absorbance at 450 nm was bleached, relative to reduction with excess sodium dithionite (not shown). In contrast, 3.8 µM XO reacted with the NADH-generating system was reduced at approximately 7 x 10$^{-4}$ min$^{-1}$. This reaction was not followed to completion; however, at 26 h the XO sample was only 61% reduced (not shown). As XO is reduced by NADH much more slowly than XDH, these experiments are consistent with the conclusion that the 95% bleaching of the XDH sample is due to reduction of the dehydrogenase form enzyme and that the oxidase form constitutes no more than 5% of the total sample.

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**TABLE I**

| Enzyme species | Observed rate constant of formation $k_{ave}$ | Extinction 450 nm | Remaining electrons |
|----------------|---------------------------------------------|-------------------|---------------------|
| Reduced XDH   | $k_{ave}$ = 72,000 ± 16,000                  | 12,900 ± 1,900    | 5.93 ± 0.87         |
| Intermediate 1| $k_1$ = 72,000 ± 16,000                      | 26,300 ± 1,200    | 1.50 ± 0.07         |
| Intermediate 2| $k_2$ = 3,500 ± 1,300                        | 29,400 ± 1,400    | 0.97 ± 0.05         |
| Intermediate 3| $k_3$ = 120 ± 21                             | 34,000 ± 700      | 0.45 ± 0.01         |
| Oxidized XDH  | $k_4$ = 16 ± 3.5                             | 37,800            | 0.00                |

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**FIG. 1.** Spectral intermediates in the reaction of fully reduced XDH with molecular oxygen. Reduced XDH (17.3 µM) was reacted with 610 µM oxygen at 25 °C, pH 7.5. Spectra were recorded from 1.25 ms to 30 min in a diode array stopped-flow instrument. Spectra are derived from a free fit to the data. Spectrum A, fully reduced XDH; spectrum B, intermediate 1; spectrum C, intermediate 2; spectrum D, intermediate 3; spectrum E, oxidized XDH.
intermediates 1 and 3 correspond to mixtures of enzyme species. With an $E_{\text{g0}}$ of 26,300 m$^{-1}$ cm$^{-1}$, intermediate 1 appears to be a mixture of 50% XDH$_{4\text{e}}$ and 50% XDH$_{3\text{e}}$, which have calculated extinction coefficients of 23,000 and 29,600 m$^{-1}$ cm$^{-1}$, respectively. Intermediate 2 has an $E_{\text{g0}}$ of 29,400 m$^{-1}$ cm$^{-1}$ which corresponds closely to that of XDH$_{3\text{e}}$. At $E_{\text{g0}}$ of 33,400 m$^{-1}$ cm$^{-1}$, intermediate 3 appears to be composed of 45% XDH$_{4\text{e}}$ and 55% XDH$_{3\text{e}}$. This analysis indicated that the number of electrons remaining in XDH at the end of the first through fourth phases is 1.50, 0.97, and 0.45, and 0.00 electrons, respectively (Table 1). Diode array data were also analyzed by a fixed fit with $k_{\text{obs}}$ values of 44, 2.1, 0.070, and 0.0095 s$^{-1}$ for the first through fourth phases, respectively, as calculated from the experimentally determined rate constants (see below). Spectra derived from this fixed fit were indistinguishable from those shown in Fig. 1. The oxidation of XDH$_{3\text{e}}$ with molecular oxygen can be described as proceeding through the species described in Scheme 1.

Kinetics of Oxidation by Molecular Oxygen—XDH (15 μM after mixing) was reduced by dithionite and then reacted in the stopped-flow instrument with solutions of phosphate buffer pre-equilibrated with oxygen, at 25 °C, pH 7.5. Data were recorded at individual wavelengths for greater kinetic resolution. Reaction traces at 610 μM oxygen are presented in Fig. 2, along with fits to the data at 450, 550, and 620 nm. There are four reaction phases apparent in the data in Fig. 2. Data at 450, 550, and 620 nm were fit (see “Materials and Methods”) with non-unique solutions, the oxygen reaction was repeated with normal traces. Agreement was precise (not shown); there-fore, photochemistry was considered to be insignificant.

As fitting reaction traces with four exponentials can lead to non-unique solutions, the oxygen reaction was repeated with samples of XDH that had been reduced to approximately the 6,-, and 2-electron states. Samples of XDH (4.5 μM for XDH$_{4\text{e}}$ and 4.7 μM for XDH$_{2\text{e}}$) were carefully photo-reduced (Fig. 4A) until the visible spectra closely resembled that of the desired species. Samples were then reacted in the stopped-flow instrument with buffer at various oxygen concentrations at 25 °C, pH 7.5. Traces of the reactions at 450 nm and 610 μM oxygen are shown in Fig. 4B. There is almost no absorbance change at 550 or 620 nm in the oxidation of XDH$_{4\text{e}}$ and XDH$_{2\text{e}}$. The reaction of XDH$_{2\text{e}}$ with oxygen required four exponentials to adequately fit, with $k_{\text{obs}}$ values at 610 μM oxygen of 48, 2.8, 0.073, and 0.0083 s$^{-1}$ for the first through fourth phases. Each phase is linearly dependent on oxygen concentration (Fig. 3) with calculated rate constants $k_1 = 76,000 \pm 26,000$ m$^{-1}$ s$^{-1}$, $k_2 = 4,500 \pm 2,500$ m$^{-1}$ s$^{-1}$, $k_3 = 110 \pm 43$ m$^{-1}$ s$^{-1}$, and $k_4 = 12 \pm 5.6$ m$^{-1}$ s$^{-1}$. The reaction of XDH$_{4\text{e}}$ with oxygen required three phases for an acceptable fit. At 610 μM oxygen, $k_{\text{obs}}$ values 8.5, 0.080, and 0.011 s$^{-1}$ were determined. Each of these three phases is also linearly dependent on oxygen concentration (Fig. 3) with rate constants of $k_2 = 13,000 \pm 2,500$ m$^{-1}$ s$^{-1}$, $k_3 = 130 \pm 43$ m$^{-1}$ s$^{-1}$, and $k_4 = 19 \pm 4.3$ m$^{-1}$ s$^{-1}$. Note that the starting points for these reactions (XDH$_{4\text{e}}$ and XDH$_{2\text{e}}$) are only approximate. Also, initial spectra represent the sum of a population of enzyme species. The observation of these kinetic phases in the oxidation of XDH$_{4\text{e}}$ supports the assignment of intermediate 1 as a significantly oxidized species.

To obtain a set of rate constants consistent with all three of these kinetic experiments, rate constants corresponding to the same reaction were averaged (Table I). Values for $k_4$ of 12 and 19 m$^{-1}$ s$^{-1}$ from the XDH$_{4\text{e}}$ and XDH$_{2\text{e}}$ experiments, respectively, were averaged to give $k_4\text{ave} = 16 \pm 3.5$ m$^{-1}$ s$^{-1}$. Excel-

![FIG. 2. Plot of absorbance versus time for the oxidation of fully reduced XDH with molecular oxygen. Dithionite-reduced XDH (15 μM) was reacted with 610 μM oxygen at 25 °C, pH 7.5, in a single-wavelength stopped-flow instrument. Reaction traces, bold curves, are overlaid with fits to the data, narrow curves. Data are presented at 450, 550, and 620 nm. Note log time scale.](image-url)
lent agreement was obtained when \( k_{\text{obs}} \) values for each oxygen concentration were calculated from \( k_{2 \text{ ave}} \), and used to fit data from the XDH\textsubscript{6e}-experiment, whose \( k_{4 \text{ ave}} \) values varied inconsistently in free fits. Values for \( k_2 \) of 110, 110, and 130 \( \text{M}^{-1} \text{s}^{-1} \) were averaged to give \( k_{2 \text{ ave}} = 120 \pm 21 \text{ M}^{-1} \text{s}^{-1} \). Values for \( k_2 \) of 2,400, 4,500, and 13,000 \( \text{M}^{-1} \text{s}^{-1} \) were first averaged to give \( k_{2 \text{ ave}} = 6,700 \pm 5,700 \text{ M}^{-1} \text{s}^{-1} \), but \( k_{4 \text{ ave}} \) values calculated from this rate constant could not be used to fit data from experiments starting with XDH\textsubscript{6e} and XDH\textsubscript{4e}. Instead, the values of \( k_2 \), from the latter two experiments were averaged to give \( k_{2 \text{ ave}} = 3,500 \pm 1,300 \text{ M}^{-1} \text{s}^{-1} \), which could be used to fit data from all three experiments. As can be seen in Fig. 4B, the amplitude of the first phase is extremely small for the reaction of XDH\textsubscript{2e} with oxygen, thus explaining the poor agreement with the other experiments. Values of \( k_1 \) of 68,000 and 76,000 \( \text{M}^{-1} \text{s}^{-1} \) were averaged to give \( k_{1 \text{ ave}} = 72,000 \pm 16,000 \text{ M}^{-1} \text{s}^{-1} \). These four average rate constants can be used together to fit all of the experimental data at all wavelengths studied.

**Influence of Desulfo Enzyme, Ligands, and Method of Reduction on the Kinetics of Oxidation**—Samples of XDH used in these studies contained approximately 25–35% inactive desulfo form. To assess the influence of this inactive XDH on the observed kinetics, desulfo XDH was prepared by prior incubation with potassium cyanide, until no measurable activity remained. Desulfo XDH (7.5 \( \mu \text{M} \) after mixing) was photo-reduced and reacted with 610 \( \mu \text{M} \) oxygen. Reaction traces (not shown) were identical in form to those of native samples. Four exponential fits with observed rates of 40, 3.2, 0.17, and 0.021 \( \text{s}^{-1} \) were required to account for the reaction traces at 450 nm. XDH was also reacted with the mechanism-based inhibitor allopurinol (19). A solution of 8.8 \( \mu \text{M} \) XDH was incubated with 1 \( \text{mM} \) allopurinol and 2.5 \( \text{mM} \) DTT for 1 h at 25 °C, pH 7.5. No detectable activity remained. The sample was desalted on Sephadex G-25 and prepared for the stopped-flow as above. Allopurinol-inhibited XDH (6.1 \( \mu \text{M} \) after mixing) was photo-reduced and reacted with 610 \( \mu \text{M} \) oxygen at 25 °C, pH 7.5. Resulting traces (not shown) were similar to that of native XDH and fit freely to \( k_1 \), values of 42, 3.7, 0.13, and 0.014 \( \text{s}^{-1} \). XDH (7.5 \( \mu \text{M} \) after mixing) photo-reduced in the presence of urate (250 \( \text{mM} \)) also reacted with 610 \( \mu \text{M} \) oxygen as normal XDH. Values of \( k_{4 \text{ ave}} \) from the four exponential fits are 31, 2.0, 0.998, and 0.011 \( \text{s}^{-1} \). As the oxidation kinetics of dithionite- and photo-reduced XDH are nearly identical (except for the extent of reaction), it is also apparent that neither the method of reduction nor sulfite derived from dithionite influences oxidation. We conclude from these experiments that alterations at the molybdenum center such as cyanoysis or binding of allopurinol, urate, or sulfite do not affect the kinetics of oxidation by molecular oxygen.

**Quantitation of Superoxide Formed in the Reaction with Molecular Oxygen**—To assess the ability of XDH to form \( \text{O}_2^\cdot \) relative to XO, and to elaborate on the mechanism in Scheme 1, the amount of \( \text{O}_2^\cdot \) formed during XDH oxidation was measured. The superoxide dismutase (SOD)-inhibited reduction of cytochrome c (cyt c) (17) has been used successfully to quantitate the amount of \( \text{O}_2^\cdot \) produced in the reaction of bovine milk XO with oxygen (12, 13). To minimize complications arising from partial re-oxidation of reduced XDH samples prior to reaction with \( \text{O}_2 \) and cyt c, and because of the slowness of the reaction, experiments were performed in anaerobic cuvettes. A solution of 6.5 \( \mu \text{M} \) XDH was photo-reduced anaerobically. The cuvette was opened, 50 \( \mu \text{M} \) cyt c was added, and the sample was then quickly equilibrated with air. Reduction of cyt c was measured at 550 nm in a diode array spectrophotometer (Fig. 5). Only about 20% of the total absorbance change occurred in the dead time of these experiments, about 30 s. The reaction exhibited biphasic kinetics with rates of 0.0074 and 0.0012 \( \text{s}^{-1} \) (Fig. 5, inset). At air saturation and 25 °C, these correspond to second-order rate constants of 29 and 4.7 \( \text{M}^{-1} \text{s}^{-1} \), respectively. Prior to anaerobiosis, XDH samples were preincubated with 500 \( \mu \text{M} \) methylmethane thiolsulfonate (MMTS) to derivatize any reactive cysteines. In the absence of MMTS treatment, reduction of cyt c on mixing with oxidized XDH was observed. Presumably these reactive cysteines can reduce cyt c. XDH assayed after MMTS treatment, reduction of cyt c on mixing with oxidized XDH was observed. Presumably these reactive cysteines can reduce cyt c. XDH assayed after MMTS treatment was not altered in xanthine/NAD or xanthine/oxygen turnover, indicating that MMTS does not inactivate XDH or convert XDH to XO. The final spectrum after re-oxidation in the absence of SOD indicated that a total of \( 4.7 \pm 0.16 \text{ eq cyt c reduced per XDH were formed. Identical reactions in the presence of } 10 \mu \text{g/ml SOD (Fig. 5) showed that } 3.0 \pm 0.14 \text{ eq cyt c reduced per XDH are formed in a superoxide-independent manner. The difference between these values, } 1.7 \pm 0.15, \text{ is the total number of mol of } \text{O}_2^\cdot \text{ per mol of XDH. Of the total eq } \text{O}_2^\cdot \text{ detected, } 1.1 \pm 0.03 \text{ are observed in the faster phase of cyt c reduction, which is consistent with the oxidation kinetics in the absence of cyt c. One explanation for the slow formation of}

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**FIG. 3.** Dependence of observed rate on oxygen concentration for the oxidation of XDH at various reduction states. XDH was photo-reduced to differing levels and then reacted with varying oxygen concentrations in a stopped-flow instrument at 25 °C, pH 7.5. Solid circles, fully reduced XDH; solid squares, 4-electron-reduced XDH; solid triangles, 2-electron-reduced XDH. Data are fit to a linear equation using the Kaldigraph program. Solid lines, fully reduced XDH; dashed lines, 4-electron-reduced XDH; -- --, 2-electron-reduced XDH. A, phase 1; B, phase 2; C, phase 3; D, phase 4.
Oxidation of Xanthine Dehydrogenase by Molecular Oxygen

**FIG. 4. Reaction of partially reduced XDH with molecular oxygen.** XDH was photo-reduced to various levels and then reacted with 610 μM oxygen in the stopped-flow instrument at 25 °C, pH 7.5. XDH at the 6- and 2-electron-reduced states were 4.69 μM, whereas XDH at the 4-electron-reduced state was 4.47 μM. Spectra were normalized by subtraction at 700 nm to correct for baseline shifts but were not corrected for concentration differences. Spectra a (---), 6-electron-reduced XDH; spectrum b, 4-electron-reduced XDH; spectrum c, 2-electron-reduced XDH; spectrum d, oxidized XDH from the experiment with 2-electron-reduced XDH. B, reaction traces at 450 nm, bold curves, overlaid with fits to the data, narrow curves. Traces were normalized by subtraction to the same end point to correct for base-line shifts. Solid curve, 6-electron-reduced XDH; (---) 4-electron-reduced XDH; dotted curve, 2-electron-reduced XDH. Note log time scale.

The remaining 0.6 eq O$_2$ is that cyt c may be forming a complex with XDH and thus be retarding the reaction with oxygen. Note that a large amount of cyt c is reduced in the presence of SOD. Increasing the concentration of SOD from 10 to 50 μg/ml had no effect on the quantity of cyt c reduced.

The anaerobic reaction between photo-reduced XDH and cyt c was measured by mixing cyt c from a side arm of an anaerobic cuvette. At 2 μM XDH, the concentration of cyt c was varied from 10 to 100 μM. Monophasic reduction of cyt c was observed with essentially no concentration dependence on the observed rate, 1.7 × 10$^{-3}$ s$^{-1}$ at 10 μM cyt c and 1.4 × 10$^{-3}$ s$^{-1}$ at 100 μM cyt c. This indicates that cyt c binds tightly to XDH. This tight binding is proposed to impede the reaction of XDH with oxygen, thus explaining the slow kinetics of superoxide-dependent cyt c reduction. Also, this binding may favor cyt c as an electron acceptor over oxygen. Clearly, the direct reduction of cyt c by XDH significantly complicates the determination of O$_2$-dependent reduction, and the measured 1.7 eq O$_2$ per XDH probably represents a lower limit of the true value.

To measure O$_2$ formation in a system more relevant to turnover, and to determine from which redox states O$_2$ is formed, oxidized XDH (4.0 μM active enzyme after mixing) was pretreated with MMTS and reacted in the stopped-flow apparatus with solutions of xanthine, 10 μg/ml catalase, 610 μM oxygen, and 50 μM cyt c with and without 20 μg/ml SOD (not shown). Experiments with 2 μM xanthine (0.5 equivalents xanthine per active XDH) yielded total absorbance changes equal to 3.9 μM cyt c, after subtraction of the SOD-independent change. This is equivalent to a total of 1.9 mol of O$_2$ per mol of xanthine. Traces were multiphasic, with 0.97 eq cyt c formed at the slowest rate of 0.0011 s$^{-1}$, significantly slower than the value of 0.0095 s$^{-1}$ predicted from $A_{450}$ of 16 M$^{-1}$ s$^{-1}$. Assuming the enzyme is reduced to only XDH$_{2e-}$, this corresponds to 1.9 total O$_2$ per XDH$_{2e-}$ or to 0.93 O$_2$ per XDH$_{2e-}$ counting only that portion consistent with the rate of oxidation in the absence of cyt c. The reaction of 4 μM active XDH with 2 μM xanthine and 610 μM oxygen was also monitored in the absence of cyt c (not shown). After an initial decrease in $A_{450}$ corresponding to reduction at 8.6 s$^{-1}$, a biphasic increase in $A_{450}$ at 0.17 and 0.018 s$^{-1}$ restored XDH$_{ox}$. These oxidation rates are reasonably consistent with those determined with pre-reduced XDH. Experiments with 8 μM xanthine (2.0 eq xanthine per active XDH) yielded 7.5 μM cyt c red after subtraction of the total SOD-independent change. 6.2 μM cyt c red was formed in that portion occurring during the time scale of XDH oxidation in the absence of cyt c. A total of 1.6 to 1.9 eq O$_2$ is formed per XDH molecule. As XDH is reduced to an oxidation state below XDH$_{2e-}$, little additional O$_2$ is formed. Due to interference from the direct reduction of cyt c by XDH, 1.9 eq O$_2$ per XDH is also considered a minimum value. This measurement of 1.9 mol of O$_2$ per mol of XDH with xanthine as the electron donor corresponds well with the 1.7 eq O$_2$ detected with photo-reduced XDH. A more conservative lower end of 0.93 to 1.1 eq represents that O$_2$ detected at a rate comparable with oxidation in the absence of cyt c. As much as 0.97 eq O$_2$ is formed at approximately 1.2 × 10$^{-3}$ s$^{-1}$. This O$_2$ production is proposed to be slow due to inhibition by cyt c binding to XDH. Binding and subsequent reduction of the cyt c may also perturb the amount of O$_2$ formed. These experiments are in agreement...
predicting the behavior toward oxygen of XDH samples at particular average redox states.

A more complete model, using the theoretical extinction coefficients, requires bifurcations to account for the mixture of intermediate states (Scheme 2). A combination of XDH$_{1e}$- and XDH$_{0e}$-has been observed to be formed in a single phase at 72,000 m$^{-1}$ s$^{-1}$ when starting with XDH$_{8e}$- and at approximately 30,000 m$^{-1}$ s$^{-1}$ when starting with XDH$_{6e}$. This requires up to 6.5 electron oxidation exhibiting overall monophasic kinetics. Attempting to unravel the elementary steps that comprise this phase would amount to speculation. Starting with XDH$_{6e}$, the model in Scheme 2 predicts that rate constant $k_1$ has a value of a least 70,000 m$^{-1}$ s$^{-1}$. Rate constants $k_2$ and $k_3$ are suggested to occur with an overall rate constant of approximately 70,000 m$^{-1}$ s$^{-1}$. The 2-fold apparent discrepancy of rates between dithionite- and photo-reduced XDH in the first step of oxidation is consistent with the different starting points, XDH$_{6e}$- and XDH$_{6e}$-, respectively, and the rate constants listed. Altogether, steps $k_1$, $k_2$, and $k_3$ are proposed to produce XDH$_{0e}$- and 3 eq H$_2$O$_2$. The XDH$_{6e}$- formed can react in another 2-electron process, $k_4$, to yield XDH$_{4e}$ and 1 mol of H$_2$O$_2$, or it can react in two consecutive 1-electron processes, $k_5$ and $k_6$, to give XDH$_{4e}$ and 2 mol of O$_2$. The values of $k_5$ and $k_6$ must be similar in magnitude to account for the oxidation state of intermediate 3. Starting with XDH$_{4e}$-, two consecutive 2-electron oxidations, $k_5$ and $k_7$, can occur with an overall rate constant of 70,000 m$^{-1}$ s$^{-1}$, resulting in XDH$_{4e}$- and 2 mol of H$_2$O$_2$. XDH$_{2e}$- is predicted to react in either 1-electron process through $k_4$ to form XDH$_{2e}$- and an equivalent of O$_2$ or in a 2-electron process through $k_5$ to yield XDH$_{4e}$- and an equivalent of H$_2$O$_2$. The rate constant $k_4$ was assigned a value of 3,000 m$^{-1}$ s$^{-1}$ to correspond with steady-state data (see “Discussion”), although simulations indicate this rate constant can be varied over a factor of 2 with little effect. The 2-electron oxidation of XDH$_{4e}$- to XDH$_{2e}$-; $k_5$, was modeled at 8,000 m$^{-1}$ s$^{-1}$ to give good correspondence with the data. From XDH$_{1e}$- a single 1-electron oxidation, $k_{11}$, would result in XDH$_{0e}$ and 1 mol of O$_2$. Scheme 2 predicts that XDH$_{1e}$ reacts with oxygen to form 1 mol of O$_2$ and 0.5 mol of H$_2$O$_2$. This is not inconsistent with experiments in which 0.93 to 1.9 eq O$_2$ are detected when XDH is reduced with [one-half] eq xanthine, forming mostly XDH$_{2e}$. The binding of cyt c to XDH has been shown to impede the rate of oxygen oxidation; this binding may also change the product distribution.

Simulating the mechanism in Scheme 2 with 67% XDH$_{6e}$- and 33% XDH$_{6e}$- as initial species and with the theoretically predicted extinctions yields a reaction trace in reasonable correspondence with the actual data (Fig. 6, curve C). Note that reactions starting with 100% XDH$_{6e}$- predict formation of 1 eq O$_2$ per XDH, and those starting with XDH$_{6e}$- predict 1.8 mol of O$_2$. This range of predicted O$_2$ stoichiometries is consistent with that observed experimentally. A mixture of starting material oxidation states is reasonable as the extinction coefficients at 450 nm of XDH$_{6e}$-, XDH$_{6e}$-, and XDH$_{6e}$- vary over only 1,200 m$^{-1}$ cm$^{-1}$. Modeling with only XDH$_{6e}$-, as the initial species results in simulations in which the extinction of intermediate 1 is far too low (not shown). This can only be rectified by adding a bifurcation at XDH$_{6e}$-, involving 1-electron oxidation to form XDH$_{6e}$- and O$_2$. Such a step is certainly possible. Decreasing rates of 2-electron oxidation, >70,000, 8,000, and 200 m$^{-1}$ s$^{-1}$, are proposed to correspond with the decreasing fraction of FADH$_2$ as the oxidation state of XDH increases.

While the fraction of FADH$_2$ varies at least 100-fold over the various redox states, the fraction of FADH only varies approximately 3-fold. The model in Scheme 2 is only approximate and is severely limited by the data available. This mechanism is
**Scheme 2. Predicted model of the oxidation of reduced XDH with molecular oxygen.** Reactions described are predicted from simulations to the data. Bifurcations were included to account for observed mixtures of redox states. Individual rate constants are given for the bifurcations.

presented to demonstrate the reactions proposed to be significant but should only be considered in a qualitative manner.

**Steady-state Kinetics of Xanthine/Oxygen and NADH/Oxygen Turnover**—Since XDH can use molecular oxygen as an electron acceptor, appreciable xanthine oxidase activity is expected. Experiments presented above estimate XO contamination to be no more than 5%, indicating this xanthine/oxygen activity is inherent to XDH. The xanthine/oxygen turnover of XDH was measured by the method of initial rates at 25 °C, pH 7.5. The sample of XDH used in this experiment was determined to be 76% active (see “Materials and Methods”). As samples contained 0.50 μM total XDH after mixing, the concentration of active XDH used was (0.50 μM)/(0.76) = 0.67 μM. A Lineweaver-Burk plot of the data shows near-parallel lines, suggestive of a ping-pong mechanism (not shown). For xanthine/oxygen turnover of XDH, k<sub>cat</sub> is 2.1 ± 0.1 s<sup>−1</sup>, K<sub>m</sub> for xanthine is 1.9 ± 0.4 μM, and K<sub>m</sub> for oxygen is 65 ± 9.0 μM. For comparison, k<sub>cat</sub> for xanthine/NAD turnover of XDH is 6.3 s<sup>−1</sup> (1), 3-fold faster. Also, at pH 7.5, 25 °C, XO has a k<sub>cat</sub> of 13 s<sup>−1</sup> (20).

As NADH can reduce XDH at a rate of 18 s<sup>−1</sup> (21) XDH is predicted to contain NADH oxidase activity. NADH oxidase activity of XDH was measured by the method of initial rates, at 25 °C, pH 7.5. A Lineweaver-Burk plot of the data shows a set of parallel lines, consistent with a ping-pong mechanism (not shown). NADH oxidase activity was determined to be independent of the fraction of desulfo XDH by assaying before and after treatment with potassium cyanide. For NADH oxidase activity, XDH has a k<sub>cat</sub> of 2.5 ± 0.9 s<sup>−1</sup>, a K<sub>m</sub> for NADH of 2.8 ± 0.5 μM, and a K<sub>m</sub> for oxygen ≥2 mM. A replot of V<sub>max</sub> (app) from the primary velocity versus NAD concentration plot, versus oxygen concentration yielded a straight line out to 610 μM oxygen with no evidence of saturation. Therefore, determination of k<sub>cat</sub> involves a very large extrapolation and is thus less accurate.

**Competition of NAD and Molecular Oxygen for Reducing Equivalents**—The ability of oxygen to compete with NAD as an electron acceptor was tested by measuring the rate of NADH formation in initial-rate assays while systematically varying the concentrations of xanthine, NAD, and oxygen (not shown). Detectable inhibition of the rate of NADH formation was only observed at high oxygen and very low xanthine or NAD concentrations. At 100% oxygen saturation, 1.2 mM at 25 °C, and at 2 μM xanthine and 14 μM NAD, 77% of the anaerobic rate was observed. K<sub>m</sub> values for xanthine and NAD are ≈1 and 7 μM, respectively (1). These xanthine and NAD concentrations are at the lower limit of our spectrophotometric detection. At xanthine and NAD concentrations equal to 8 and 56 μM, respectively, 91% of the anaerobic rate is observed. Under initial-rate conditions, oxygen can only successfully compete with NAD as a substrate for XDH at NAD or xanthine concentrations significantly below each K<sub>m</sub>.

These experiments with xanthine, NAD, and oxygen were also performed with an oxygen electrode, monitoring the disappearance of molecular oxygen (not shown). The oxygen electrode is not sensitive enough to demonstrate inhibition of oxygen consumption by NAD during the initial-rate portion of the reaction; therefore, the entire reaction was followed. Experiments were performed at 500 μM xanthine. At concentrations of NAD from 0 to 1 mM, all of the oxygen (260 μM) was eventually consumed over a time course that ranged from 15 to 50 min. The time required for complete oxygen depletion was proportional to the NAD concentration. This result can be simply explained as consumption of oxygen by the NADH oxidase activity of XDH once the xanthine/NAD reaction has gone to completion. This conclusion is supported by the observation of a plateau and a decrease in 340 nm absorbance when aerobic xanthine/NAD assays are followed at longer times. The significance of this observation is that electron equivalents from xanthine can ultimately be transferred to oxygen in the presence of NAD and NADH. Although oxygen competes poorly for electron equivalents from XDH when there is an excess of NAD present, the NAD is eventually consumed. Although disfavored kinetically, presumably oxygen is the ultimate electron acceptor due to the high redox potential of the O<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> couple, +300 mV at pH 7.0 (22), much higher than that of the NAD/NADH couple, −320 mV at the same pH.

**DISCUSSION**

A simplified model for the oxidative half-reaction of bovine milk XDH with molecular oxygen is presented in Scheme 1. This mechanism describes oxidation as proceeding by four irreversible second-order reactions with oxygen, although these are certainly complex combinations of rate constants. Using the reduction of cyt c as an indicator for O<sub>2</sub> at least 1.9 mol of O<sub>2</sub> are formed in the conversion of XDH<sub>2e−</sub> to XDH<sub>ox</sub>. Thus, O<sub>2</sub> is produced from the last electrons to be oxidized. The rate constants calculated from the data decrease by over 3 orders of magnitude from the start to the end of the reaction. It is proposed that these changes in rate may be due to a decrease in electron density at the FAD center, the site at which oxygen reacts. The inherent reactivity of reduced XDH toward oxygen may also decrease as its oxidation state changes, and the redox potential of the enzyme becomes more positive. Two different types of flavin reactivity can be distinguished. The reaction of FADH<sub>2</sub> with oxygen to produce FAD and H<sub>2</sub>O<sub>2</sub> is thought to be fast, while the reaction of FADH<sub>2</sub> with oxygen to produce FAD and O<sub>2</sub> is slow. The redox potentials predict that FADH<sub>2</sub> is
present significantly only at XDH$_{2e}$, and lower, while a large fraction of FADH$_2$ persists from XDH$_{4e}$ to complete oxidation. Superoxide is not formed until the higher oxidation states because the reaction of oxygen with FADH$_2$ is proposed to be much faster than with FADH$_1$, even when only a small fraction of FADH$_2$ is present. At XDH$_{2e}$ and XDH$_{1e}$, very little FADH$_2$ is predicted to be present, thus favoring the slower reaction to make O$_2$.

There is substantial precedent for altered reactivity of flavin hydroquinone and semiquinone species toward molecular oxygen. Such behavior has been shown in the oxidation of the simpler flavoproteins glucose oxidase from Aspergillus niger (25) and flavodoxin from Megasphaera elsdenii (26, 27). Experiments with chicken liver XDH were consistent with such selectivity of reaction rates with oxygen, with hydroquinone reacting at 1,900 M$^{-1}$ s$^{-1}$ and semiquinone at 260 M$^{-1}$ s$^{-1}$, at pH 7.8, 4 °C (14). The overall oxidative half-reaction of bovine milk XDH is suggested to proceed by at least four second-order reactions, controlled by the electron distribution within XDH and by the redox potentials of the enzyme. Equilibria controlling electron distribution are expected to be maintained quickly from studies on intramolecular electron transfer (23, 24) and the rapid equilibrium hypothesis of Olsson et al. (28).

The current results share some similarities to those determined in the oxidative half-reaction of bovine milk XO. Those experiments were carried out at 25 °C, pH 8.5, and were proposed to follow a four-step reaction sequence: XDH$_{4e}$ → XDH$_{3e}$ → XDH$_{2e}$ → XDH$_{1e}$ → XDH$_{0e}$ (12, 13). Three kinetic phases at 450 nm were observed, a short lag phase, a saturating phase with a limiting rate of 125 s$^{-1}$, and an apparent $K_d$ of 500 μM, and a phase directly proportional to oxygen concentration with a second-order rate constant of 10,000 M$^{-1}$ s$^{-1}$. At the end of the second phase, spectral changes indicated that XO had been oxidized by 5 electron equivalents. Each research group that has studied the reaction of XO with oxygen has proposed the formation of a binary complex between XO$_{red}$ and oxygen based on the observation of saturation kinetics (11–13). The reaction of most known reduced flavoproteins with oxygen proceeds via an irreversible second-order reaction (29), and the current data certainly support this in the case of bovine milk XDH. It is possible that the reaction of XO with oxygen also proceeds via purely bi-molecular reactions. Saturation kinetics could be observed due to complications arising from a series of chemical reactions that are exhibited in a single spectrophotometric phase.

The oxidation of chicken liver XDH is quite different from that of bovine XDH. The chicken liver enzyme reacts with oxygen according to a model similar to that of XO, only with a bifurcation at XDH$_{4e}$ to form XDH$_{3e}$ in either a single 2-electron step or in two consecutive 1-electron steps (14). Between 2.8 and 3.0 mol of O$_2$ were detected with the chicken liver enzyme. With bovine milk XDH, the rate at which FADH$_2$ reacts with oxygen is much slower than in the chicken liver enzyme (260 M$^{-1}$ s$^{-1}$ (14)) and cannot compete with the 2-electron rate until the fraction of FADH$_2$ is less than 0.01, which is at XDH$_{3e}$ or higher. This explains why more O$_2$ is detected in the oxidation of the chicken liver enzyme. For all three of the enzymes discussed, the reaction with oxygen would appear to involve a series of bi-molecular collisions, with the reaction rate and products at least partially a function of the type of reduced flavin encountered.

If reduced bovine milk XDH can react with oxygen at least as fast as 72,000 M$^{-1}$ s$^{-1}$, then how can NAD effectively compete as an electron acceptor? It has been proposed previously that during xanthine/NAD catalysis, XDH cycles between the 2- and 4-electron-reduced states (21). XDH$_{4e}$ would thus be the species for which NAD and oxygen compete. Reduced XDH reacts with NAD at a limiting rate of 170 s$^{-1}$, subsequent to rapid binding. According to Scheme 1, XDH$_{4e}$ reacts with oxygen with a rate constant of at least 72,000 M$^{-1}$ s$^{-1}$, which at air saturation would give a $k_{cat}$ of 18 s$^{-1}$, much less than 170 s$^{-1}$ for the NAD reaction. In addition, NAD appears to bind very rapidly, with an estimated association rate constant of at least 1 × 10$^7$ M$^{-1}$ s$^{-1}$, 140-fold greater than the fastest observed oxygen reaction. For comparison, at 200 μM oxygen XO is estimated to react at 35 s$^{-1}$, at pH 8.5 (12). At its lowest oxidation states, XDH reacts with oxygen at rates comparable with those of XO. At the higher oxidation states the rates are much slower than the slow phase of the XO reaction, 16 M$^{-1}$ s$^{-1}$ versus 1 × 10$^4$ M$^{-1}$ s$^{-1}$ (12). This difference is proposed to be due in part to the greater stabilization of FADH$^+$ with respect to FADH$_2$ in XDH; the semiquinone form appears to react much slower than the hydroquinone. In addition, there must be a significant (630-fold) reduction in the rate at which the semiquinone of XDH reacts with respect to that of XO. Not only is the oxygen reaction retarded by the distribution of electrons in XDH, but the inherent reactivity of the flavin semiquinone is much smaller.

XDH contains appreciable xanthine/oxygen and NADH/oxygen activities. The $k_{cat}$ values for these reactions are very close, 2.1 ± 0.1 and 2.5 ± 0.9 s$^{-1}$, respectively, and constitute 33 and 40% of the $k_{cat}$ value for xanthine/NAD catalysis, respectively. Since the reductive half-reaction in both cases has been shown to be faster than 2.5 s$^{-1}$ (21), the reaction with oxygen must greatly limit turnover for both. The spectrum of XDH during xanthine/oxygen catalysis approximates XDH$_{3e}$ (1); the rate-limiting step is oxidation, and the spectrum represents the more reduced form. XDH is therefore thought to cycle between XDH$_{2e}$ and XDH$_{1e}$ during xanthine/oxygen turnover. According to Scheme 2, XDH$_{2e}$ decays at $k_{red}$ of 7.0 s$^{-1}$ and $k_{cat}$ of 2.8 s$^{-1}$, a $k_{cat}$ of 2.0 s$^{-1}$ can be calculated which agrees quite well with the experimental value of 2.1 s$^{-1}$. XDH$_{2e}$ can either be oxidized directly to XDH$_{1e}$ by $k_{red}$ to form 1 mol of H$_2$O or it can proceed through $k_8$ and $k_9$ to form 2 eq O$_2$ via XDH$_{2e}$. The ratio of $k_8$ to $k_9$, 0.38, is consistent with the fraction of electrons from xanthine that form O$_2$ during xanthine/oxygen turnover, 0.42 (1). Although XDH produces no more O$_2$ than XO during the oxidative half-reaction with oxygen, much more O$_2$ is produced during XDH-catalyzed xanthine/oxygen turnover because it cycles through the higher oxidation states where O$_2$ is formed. NADH/oxygen turnover is proposed to cycle through XDH$_{3e}$ and XDH$_{1e}$ as well. Using $k_{red}$ of 18 s$^{-1}$ (21) and $k_{cat}$ of 2.8 s$^{-1}$, the calculated $k_{cat}$ of 2.3 s$^{-1}$ agrees well with the experimental value of 2.5 s$^{-1}$. Under initial-rate conditions, steady-state experiments performed in the presence of xanthine, NAD, and oxygen indicate that oxygen can only effectively compete at very high concentrations (100%), and only at concentrations of xanthine and NAD below their $K_m$ values, ≤1 and 7 μM, respectively (1).

McCord (30) has suggested that conversion of XDH to XO in vivo may be responsible for production of H$_2$O$_2$ and O$_2$ in postischemic reperfusion injury. Results from the current work suggest that the mammalian bovine milk XDH is itself capable of producing such reactive oxygen species, albeit at 16% the rate of XO (calculated as the ratio of $k_{cat}$ values 2.1 and 13 s$^{-1}$).
(20) at 25 °C, pH 7.5). Estimates of relative amounts of XO and XDH based solely upon turnover assays must therefore be made with caution, as XDH contains an intrinsic xanthine oxidase activity. Monitoring the reaction with an oxygen electrode, oxygen was consumed in aerobic xanthine/NAD assays over a 15–50-min time scale from 0 to 1 mM NAD. Once xanthine/NAD turnover has gone to completion, XDH can function as an NADH oxidase, even in the presence of high NAD levels. Depletion of the amount of NAD available to XDH, relative to oxygen, should be sufficient for XDH-catalyzed production of oxygen radicals.

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