The reaction between reduced xanthine dehydrogenase (XDH) from bovine milk and NAD has been studied in detail. An understanding of this reaction is necessary for a complete description of XDH turnover with its presumed natural electron acceptor and to address the preference of XDH for NAD over oxygen as a substrate. The reaction between pre-reduced XDH and NAD was studied by stopped-flow spectrophotometry. The reaction was found to involve two rounds of oxidation with 2 eq of NAD. The first round goes to completion, and the second round reaches a slightly disfavored equilibrium. Rapid binding of NAD with an apparent $K_d$ of $25 \pm 2 \mu M$ is followed by NAD reduction at a rate constant of $130 \pm 13 \text{s}^{-1}$. NADH dissociation at a rate constant of $42 \pm 12 \text{s}^{-1}$ completes a round of oxidation. These steps have been successfully tested and modeled to repeat themselves in the second round of oxidation. The association rate constant for NAD binding was estimated to be much greater than any rate constant measured in the oxidation by molecular oxygen, thus explaining how NAD binds to XDH catalysis can be separated into a reductive half-reaction, in which two electrons at a time are transferred from xanthine to the enzyme, and an oxidative half-reaction, in which electrons are conveyed from XDH to either NAD or oxygen. The functional difference between XO and XDH is the pre-reduced substrate in the oxidative half-reaction. The current study investigates the oxidative half-reaction of XDH with NAD. Characterization of the reaction between XDH and its primary substrate in the oxidative half-reaction. The current study investigates the oxidative half-reaction of XDH with NAD. Characterization of the reaction between XDH and its presumed natural electron acceptor is necessary for a complete understanding of XDH turnover. Since XO and XDH share the same protein constituents, but differ with respect to their conformation, the mechanism of this reaction can yield a better understanding of how the protein environment can influence the reactivity of a bound flavin cofactor. Of particular interest is how the reactivity of reduced XDH with NAD compares to that of molecular oxygen.

**MATERIALS AND METHODS**

Xanthine dehydrogenase was purified by the method of Hunt and Massey (4). Concentrations are expressed 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 set of experiments by determining the fraction of absorbance lost at 450 nm within 1 min on anaerobic reduction by 200 $\mu$M xanthine relative to the total absorbance change after further reduction with an excess of sodium dithionite. Samples were used were typically between 65 and 75% active. XDH activity was also routinely checked by measuring the formation of NADH at 340 nm in an air-saturated enzyme containing 100 $\mu$M xanthine, 500 $\mu$M NAD, and a catalytic (10–50 nm) amount of XDH. Percent functional enzyme was measured prior to each set of experiments by determining the fraction of absorbance lost at 450 nm within 1 min on anaerobic reduction by 200 $\mu$M xanthine relative to the total absorbance change after further reduction with an excess of sodium dithionite. XDH catalysis can be separated into a reductive half-reaction, in which two electrons at a time are transferred from xanthine to the enzyme, and an oxidative half-reaction, in which electrons are conveyed from XDH to either NAD or oxygen. The functional difference between XO and XDH is the preferential substrate in the oxidative half-reaction. The current study investigates the oxidative half-reaction of XDH with NAD. Characterization of the reaction between XDH and its presumed natural electron acceptor is necessary for a complete understanding of XDH turnover. Since XO and XDH share the same protein constituents, but differ with respect to their conformation, the mechanism of this reaction can yield a better understanding of how the protein environment can influence the reactivity of a bound flavin cofactor. Of particular interest is how the reactivity of reduced XDH with NAD compares to that of molecular oxygen.

**Calculation of Extinction Coefficients**—At 450 nm, extinction coefficients used in units of M$^{-1}$ cm$^{-1}$ are as follows: 37,800 for XDHred, with extinction increases of 7,000 per 2Fe/2S center, 12,200 for XDHox, with extinction increases of 7,000 per 2Fe/2S center, 12,200 for XDHred, 12,200 for XDHox, and a catalytic (10–50 nm) amount of XDH. Identical rates are obtained if the assay is anaerobic. Reactions were performed in temperature-equilibrated cuvettes at 25 °C. Oxidase activity was also determined to detect any spontaneous conversion to the oxidase form. Oxidase assays measure the formation of urate at 295 nm in a mixture as above, minus NAD. Xanthine dehydrogenase samples were incubated with 2.5 mM dithiothreitol for 1 h at 25 °C prior to use. Dithiothreitol was removed by gel filtration before experimentation. Argon (Matheson purity) was purchased from Matheson. All reactions were performed in 50 mM potassium phosphate, pH 7.5, 0.3 mM EDTA, and at 25 °C. Anaerobic, enzyme reduction, and rapid reaction kinetics and data analyses were performed as described previously (5).
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XDH日常工作和与需要有3,400 per 2Fe/2S center and 5,000 for FADH. Using these values and the redox potentials (9, 11) to predict the fraction of each center reduced at various oxidation states, extinction coefficients were calculated to be 16,800 M⁻¹ cm⁻¹ for XDH₂ and 23,000 M⁻¹ cm⁻¹ for XDH₂ at 450 nm and 11,200 M⁻¹ cm⁻¹ for XDH₂ at 550 nm.

Preparation of Desulfo XDH—The very closely related xanthine oxidase is known to be inactivated by cyanolysis of the labile molybdenum-site sulfur (13). Desulfo XDH was prepared by incubating a 200 μM solution of XDH with 35 mM potassium cyanide at 25 °C, pH 7.5, until no 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 previously (5).

Steady-state Rate Equation—A rate equation was derived by the King-Altmann method (14) for the ping-pong mechanism described in Scheme 2. Product binding was assumed to be zero under initial rate conditions. The following rate Equation 1 was derived in which X is Scheme 2.

\[
e^{- \frac{k_{h}k_{f}k_{j}}{k_{h}k_{f}k_{j}k_{j}}} = \frac{k_{h}k_{f}k_{j}k_{j}}{k_{h}k_{f}k_{j}k_{j}} = \frac{k_{h}k_{f}k_{j}k_{j}}{k_{h}k_{f}k_{j}k_{j}} (\text{Eq. 1})
\]

\[
k_{int} = \frac{k_{h}k_{f}k_{j}k_{j}}{k_{h}k_{f}k_{j}k_{j}} = \frac{k_{h}k_{f}k_{j}k_{j}}{k_{h}k_{f}k_{j}k_{j}} (\text{Eq. 2})
\]

\[
K_{n,NAD} = \frac{k_{h}k_{f}k_{j}k_{j}}{k_{h}k_{f}k_{j}k_{j}} (\text{Eq. 3})
\]

RESULTS

Kinetics of Oxidation by NAD—To study the reaction of reduced XDH with NAD, dithionite-reduced XDH was mixed anaerobically with NAD in the stopped-flow apparatus. Note that the visible spectrum of XDH only reflects the redox state of the iron-sulfur and flavin centers; spectral changes in the molybdenum center are minor. The reaction was monitored at various wavelengths, from 305 to 650 nm. In the experiment presented in Fig. 1, 11 μM XDH was reacted with 500 μM NAD (concentrations after mixing) at 25 °C, pH 7.5. Amplitude values from exponential fits to the data were used to construct the spectrum of the intermediate enzyme species (Fig. 1). When monitoring the reaction at 450 nm, two reaction phases are observed (Fig. 2). The first reaction phase is only observed in the 350–500 nm (Fig. 1) range and from analysis of a number of experiments has an average extinction increase at 450 nm of 9,400 M⁻¹ cm⁻¹. The extinction change at 450 nm is lower than the anticipated 12,200 M⁻¹ cm⁻¹ (12) and may be due to a FAD:NADH charge-transfer complex. However, the extinction changes due to the flavin are not known precisely. This step has been attributed to oxidation of FADH₂ to FAD, with concomitant reduction of NAD. This conclusion is supported by the difference spectrum computed by subtracting the spectrum of reduced XDH from that of the intermediate; this spectrum has maxima at approximately 380 and 445 nm, typical of oxidized flavins. Note that NADH absorbance at 340 nm does not appear to form until the second step. This apparent discrepancy is discussed below (see “Appearance of Reduced Pyridine Dinucleotide”). The observed rate constant of the first phase at 450 nm is hyperbolically dependent on NAD concentration (Fig. 3A), consistent with a model involving a first-order process preceded by a reversible second-order binding reaction (15). A hyperbolic fit yielded a limiting rate constant of 170 ± 4 s⁻¹ and an apparent Kₘ of 25 ± 2 μM. The second step observed at 450 nm corresponds to an extinction increase of 2,700 M⁻¹ cm⁻¹ and is characterized by a maximal rate constant of 16 ± 1 s⁻¹ at 50 μM NAD (Fig. 3B).

The absorbance changes associated with this phase are most consistent with a redistribution of electrons from molybdenum and iron centers to the flavin to form a significant amount of FADH⁺ and oxidized iron centers. The observed rate constant of this phase increases proportionately with NAD concentration, reaching a maximum at 50–100 μM NAD (Fig. 3B). Following this plateau, excess NAD inhibition is observed. These data have been displayed with a hyperbolic fit to emphasize the non-hyperbolic behavior. This unusual concentration dependence suggests non-productive NAD binding and will be discussed more thoroughly below. These data are consistent with a mechanism in which rapid NAD binding and reduction (reactions k₇/k₈ and k₉/k₈ in Scheme 1), observed as an absorbance increase in the flavin region, is followed by slower release

![Fig. 1. Spectral intermediates in the oxidation of fully reduced XDH by excess NAD](image1)

![Fig. 2. Plot of absorbance and fluorescence versus time for the oxidation of fully reduced XDH with NAD](image2)
of NADH (reaction $k_d/k_g$) with concomitant re-arrangement of electrons according to the redox potentials of the various centers (Scheme 1).

The reaction of XDH with NAD was monitored from 305 to 650 nm. A monophasic reaction was observed at all wavelengths outside of 350–500 nm. These data can be fit to a single exponential equation. A reaction trace at 550 nm is shown (Fig. 2). Extensive data were also collected at 340 (Fig. 5A) and 620 nm (not shown). These data are identical in form to those at 550 nm but with different amplitudes. Observed rate constants varied hyperbolically with NAD concentration (Fig. 3B) having a limiting value of $33 \pm 3 \text{s}^{-1}$ and an apparent $K_d$ of $19 \pm 2 \text{M}$. As these spectral changes are consistent with the formation of species D in Scheme 1, this step could also be involved with product release. Its hyperbolic kinetics would then be simply a consequence of the preceding step not occurring at its saturated rate.

**NADH Fluorescence**—To gain insight into the formation and release of NADH independently of the chlorophores of XDH, reduced XDH was reacted with NAD in a fluorescence stopped-flow apparatus. XDH itself undergoes no fluorescence changes on oxidation, as determined by mixing reduced XDH with oxygen (not shown). Samples were excited at 340 nm, and emission was observed from 360 nm and above. Reaction of XDHred with NAD yielded a biphasic fluorescence increase (Fig. 2). The slower phase (not shown) increased in rate and amplitude with increasing slit width and has been dismissed as a photochemical artifact, whereas the first phase was indifferent to the amount of light. The observed rate constant of the first step in the reaction is hyperbolically dependent on NAD concentration with a limiting rate of $31 \text{s}^{-1}$ and an apparent $K_d$ of 13 μM. These values agree very well with those determined by absorbance methods at 340 and 550 nm. By comparison with a standard curve, the amplitude of the first phase corresponds to 1.0 ± 0.2 mol of NAD per mol of XDH (not shown). This stoichiometry is consistent with that determined by absorbance methods (16). Since a faster phase corresponding to the oxidation of $\text{FADH}_2$ to FAD has been observed, and since the FAD center is known to be the site at which NAD and NADH interact with XDH (16), the fluorescence increase at $31 \text{s}^{-1}$ most likely corresponds to NADH dissociation. Presumably fluorescence of enzyme-bound NADH is quenched by intermediate reduction states of the enzyme. Thus, the measured 1.0 eq of NADH per XDH reflects the unbound NADH and not the total NADH.

Attempts to measure NADH quenching by mixing reduced XDH with NADH failed to show any fluorescence change, and mixing oxidized XDH with NADH results in reduction of the enzyme.

**Oxidation by Sub-stoichiometric NAD**—To determine the events associated with the reaction of reduced XDH with the first equivalent of NAD encountered, XDH was reacted with a sub-stoichiometric amount of NAD. Dithionite-reduced XDH (4.5 μM, final) was mixed anaerobically with 2.0 μM NAD (final) in the stopped-flow instrument (Fig. 4). This corresponds to 0.44 mol of NAD per mol of XDH. A biphasic reaction trace was observed (Fig. 4, inset) with observed rate constants of 28 and 4.9 s$^{-1}$ for phases one and two, respectively. At 550 nm, a single reaction phase was observed (not shown) with an observed rate constant of 4.1 s$^{-1}$. Slow rates are expected as 2 μM NAD is far below the apparent $K_d$ of 25 μM. The biphasic kinetics at 450 nm are consistent with oxidation of FAD, followed by slower NADH dissociation and electron reorganization. It is possible that biphasic kinetics are actually due to the reaction being performed under second-order conditions, although rapid binding such as that modeled in Scheme 1 would eliminate this complication. The rate constants for the product release step at 450 and 550 nm are identical when 1 eq of NAD reacts, and the observed rate constant at 450 nm is less than that at 550 nm when excess NAD reacts, consistent with two rounds of oxidation being observed only in the 450-nm region. This is because there are essentially no spectral changes below 350 nm and above 500 nm following the first round of NAD oxidation (Figs. 2 and 4). A model consistent with these results is presented in Scheme 1 and is discussed more thoroughly below.

**Oxidation of Partially Reduced XDH**—During turnover with xanthine and NAD, XDH is thought to cycle primarily through the 2- and 4-electron-reduced states (16). The reaction of XDHor− with NAD is therefore more catalytically relevant, although more difficult to study. For this reason, and also to test further the mechanism in Scheme 1, XDH (7.5 μM after mixing) was photo-reduced to approximately XDHor− and mixed anaerobically with NAD in the stopped-flow instrument. Spectra are presented in Fig. 4 of the partially reduced enzyme before (spectrum C) and after (spectrum D) mixing with 500 μM NAD. Note from the spectra that there is essentially no change in the visible spectrum at 550 and 620 nm, consistent with spectral changes at longer wavelengths corresponding to only the first round of NAD oxidation. A reaction trace at 450 nm is presented (Fig. 4, inset). This trace required two exponentials for an adequate fit, with observed rate constants of 140 and 13 s$^{-1}$ for the first and second phases, respectively. These values agree reasonably well with those determined with fully reduced XDH at 500 μM NAD of 170 and 12 s$^{-1}$. A thorough investiga-
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Scheme 1. Mechanism for oxidation of reduced XDH with NAD. Redox states given are the major resonance forms. Redox potentials of species are from the literature (9, 11). Binding reactions were made rapid equilibria such that the ratio of $k_{dissociation}$ to $k_{association}$ equals $K_d$, and both $k_{association}$ and $k_{dissociation}$ are at least 5-fold greater than the subsequent forward rate constant. The $K_d$ for NADH binding was estimated solely from simulations. The $K_d$ for NADH binding to species D (step $k_{d}$/ $k_{a}$) was estimated solely from simulations. Rate constants assigned are $k_1 = 4.0 \times 10^6$ M$^{-1}$ s$^{-1}$; $k_2 = 1.0 \times 10^6$ s$^{-1}$; $k_3 = 130$ s$^{-1}$; $k_4 = 0.083$ s$^{-1}$; $k_5 = 33$ or 50 s$^{-1}$; $k_6 = 6.0 \times 10^6$ or $9.1 \times 10^6$ M$^{-1}$ s$^{-1}$; $k_7 = 5.0 \times 10^6$ M$^{-1}$ s$^{-1}$; $k_8 = 1,000$ s$^{-1}$; $k_9 = 1,000$ s$^{-1}$; $k_{10} = 8,300$ s$^{-1}$; $k_{11} = 4.0 \times 10^6$ M$^{-1}$ s$^{-1}$; $k_{12} = 1,000$ s$^{-1}$; $k_{13} = 130$ s$^{-1}$; $k_{14} = 42$ s$^{-1}$; $k_{15} = 33$ or 50 s$^{-1}$; $k_{16} = 6.0 \times 10^6$ or $9.1 \times 10^6$ M$^{-1}$ s$^{-1}$; $k_{17} = 1.0 \times 10^6$ M$^{-1}$ s$^{-1}$; $k_{18} = 1,000$ s$^{-1}$.

Fig. 4. Reaction of fully reduced XDH with substoichiometric NAD and of partially reduced XDH with excess NAD. Experiments were performed in the stopped-flow instrument at 25 °C, pH 7.5. Spectrum A, circles, 4.5 μM fully reduced XDH; spectrum B, diamonds, 10 s after mixing 4.5 μM fully reduced XDH with 2.0 μM NAD; spectrum C, squares, 7.5 μM XDH photo-reduced to XDH$_{4e}$, normalized to 4.5 μM; spectrum D, inverted triangles, 10 s after mixing 7.5 μM XDH$_{4e}$ with 500 μM NAD; normalized to 4.5 μM; spectrum E, triangles, 20 min after mixing 4.5 μM fully reduced XDH with 610 μM oxygen. Inset, reaction traces at 450 nm. Lower trace, 4.5 μM fully reduced XDH mixed with 2.0 μM NAD; upper trace, 7.5 μM XDH$_{4e}$ mixed with 500 μM NAD, normalized to 4.5 μM.

The concentration dependence of this reaction was not possible due to difficulties with oxygen contamination and maintaining the sample at the same oxidation state. Note that the XDH$_{4e}$ starting material for this reaction is merely an average of all species; XDH in other oxidation states is certainly present. This may explain why the rate of the second phase is less than that expected (~30 s$^{-1}$ at 500 μM NAD); this reaction still shows contributions from XDH undergoing two rounds of oxidation. Also, the total signal change is quite small, thus preventing an accurate analysis. Mixing XDH$_{4e}$ with NAD resulted in no NADH fluorescence, consistent with the 1.0 mol of NADH per mol of XDH stoichiometry reported above (see "NAD Fluorescence").

Appearance of Reduced Pyridine Dinucleotide—As noted above, the spectra in Fig. 1 appear to indicate no absorbance due to NADH until the step after oxidation of the FAD. Also, a trace at 340 nm of the reaction between reduced XDH and NAD exhibits a monophasic absorbance increase (Fig. 4A) at 27 s$^{-1}$. Due to corrections for the large ultraviolet absorbance of protocatechuate (used with protocatechuate dioxygenase to maintain an anaerobic environment), data recorded at 340 nm are on the edge of the reliable region. Using spectra of Megasphaera elsdenii flavodoxin (18) as a model for the flavin in XDH, we predict a decrease in absorbance at 340 nm on two-electron oxidation of FADH$_2$. From simulations (see below), an extinction decrease of the flavin at 340 nm on two-electron oxidation of FADH$_2$ is sufficient to give the observed monophasic behavior. Thus flavin oxidation and NAD reduction can be modeled to occur simultaneously.

To test this model, reduced XDH was reacted with excess thionicotinamide adenine dinucleotide (TNAD) in the stopped-flow apparatus. TNAD has a larger extinction change on reduction than NAD, and when reduced it has a maximum at 395 nm, a region which can be observed more accurately than 340 nm. TNAD, 500 μM, reacted with photo-reduced XDH in two phases having observed rate constants of 310 s$^{-1}$ and 14 s$^{-1}$, in
Influence on the rate of oxidation by NAD.

To determine if binding of urate, the product of xanthine oxidation, has any effect on the NAD reaction, XDH was photoreduced along with urate and then reacted anaerobically with NAD. Another ligand to the molybdenum center, oxypurinol, was tested for its effect on oxidation by NAD. Enzyme oxidation of allopurinol results in conversion to oxypurinol and the Mo(IV) state of the enzyme (19). Oxypurinol binds extremely tightly to the Mo(IV) species thus formed. XDH was incubated with excess allopurinol until no activity remained. Desalting on a Sephadex G-25 column was performed to remove excess allopurinol. Urate and allopurinol each had no effect on the reaction of reduced XDH with NAD. These experiments demonstrate that ligands bound to the molybdenum center such as urate, oxypurinol, or sulfite (derived from dithionite) do not influence oxidation by NAD. These results are significant because they demonstrate that oxidation of the 2Fe2S and FAD centers of XDH can occur independently of chemistry at the molybdenum site. Also, since the same results were obtained with XDH samples reduced photochemically or by sodium dithionite, these experiments indicate the absence of possible artifacts from the method of reduction.

Mechanism and Simulation of Reaction—A model for the oxidative half-reaction of XDH with NAD is presented in Scheme 1. Binding of NAD to reduced XDH is assumed to be a rapid equilibrium process, as the data in Fig. 3A fit nicely to a rectangular hyperbola with a zero y intercept. An apparent $K_d$ of 25 $\mu M$ has been assigned to this step from the hyperbolic fit. NAD association and dissociation rate constants used in simulations, $4 \times 10^7 M^{-1} s^{-1}$ and 1,000 $s^{-1}$ respectively, are lower limits within the condition that they describe a $K_d$ of 25 $\mu M$ and are rapid with respect to $k_3$. Following NAD binding, an increase in absorbance from 350 to 500 nm is consistent with the oxidation of FADH$_2$ to FAD with concomitant reduction of NAD (step $k_3/k_4$ in Scheme 1). The observed extinction increase at 450 nm from species A to C is only 9,400 $M^{-1} cm^{-1}$, less than the 12,200 $M^{-1} cm^{-1}$ expected from oxidation of FADH$_2$ to FAD (19). Species C is proposed to be an NADH to FAD charge-transfer complex. Such a complex has been observed in the reverse reaction (16), and the relative change in $\epsilon_{450}$ is consistent with that of other flavoproteins containing similar complexes (20). Although the extrapolated rate for formation of species C was measured to be 170 $s^{-1}$ (Fig. 3A), the value of 130 $s^{-1}$ was more consistent with simulations to the data. Setting $k_3$ and (and $k_{13}$) to 130 $s^{-1}$ in simulations yielded theoretical curves whose analysis resulted in a limiting rate of 170 $s^{-1}$. Such apparent discrepancies are reasonable with a model of such complexity as that proposed. Slow dissociation of NADH followed by rapid re-equilibration of electrons within XDH forms species D, along with 1 eq of NAD. This reaction has been simulated with the experimentally determined forward rate constant, $k_4$ (and $k_{15}$), of 33 $s^{-1}$ as well as the value of 50 $s^{-1}$. While modeling with an NAD dissociation rate constant of 33 $s^{-1}$ fits well to data at 340 and 450 nm (Fig. 5), the value of 50 $s^{-1}$ results in simulations more consistent with the data at 550 nm (Fig. 5). Studies of the reduction of XDH by NADH indicate that NADH concentrations as low as 17 $\mu M$ have no influence on reaction rate with XDH$_{red}$ (16). Using 5 $\mu M$ as an upper limit for the $K_d$ of NADH, and assuming this $K_d$ is not dependent on the oxidation state of XDH, a lower limit of $6 \times 10^6 M^{-1} s^{-1}$ was used for the association rate constant of NADH, $k_{12}$ (and $k_{16}$), coupled with a dissociation constant ($k_5$ and $k_{17}$) of 33 $s^{-1}$. Alternatively, an association rate constant of 9.1 $\times 10^6 M^{-1} s^{-1}$ was used with the dissociation rate constant of 55 $s^{-1}$.

Following NADH release, electrons within XDH$_{red}$ are thought to rapidly re-equilibrate according to the redox poten-
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At the end of the reaction, the overall equilibrium to produce 2.0 eq of unbound NADH, whereas only 1.0 ± 0.2 is observed.

The model described in Scheme 1 was simulated in its entirety using Program A (Fig. 5), the use of which has been described previously (5). Program A is a data analysis and simulation program developed in the laboratory of Dr. David P. Ballou at the University of Michigan. Concentrations, rate constants, and extinction coefficients are given in the legends to Fig. 5 and Scheme 1. Two cases are presented in which the rate constants of NADH dissociation, $k_g$ and $k_{10}$, are either 33 or 50 s$^{-1}$. Simulations with the rate constant 50 s$^{-1}$ clearly result in closer correspondence with the data at 550 nm and slightly worse correspondence at 340 and 450 nm. The rate constant for NADH dissociation was taken to be the average of these two values, 42 ± 12 s$^{-1}$. Variation of NAD concentration in the simulation resulted in a series of curves whose analysis recapitulated the experimentally determined rate and binding constants within 10% error. Although no amount of modeling can prove the veracity or uniqueness of a model, these simulations are at least consistent with the mechanism of NAD oxidation presented in Scheme 1. It should be emphasized that the simulations to the model in Scheme 1, involving a large number of rate constants and intermediates with different spectroscopic properties, yield reaction traces that exhibit the biphasic kinetics observed at some wavelengths (350–500 nm) and the monophasic kinetics measured throughout the rest of the spectrum.

DISCUSSION

An interesting phenomenon in the reaction of reduced XDH with NAD is that following what is presumably hydride transfer from FADH$_2$ to NAD, electrons from the metal centers of XDH do not re-equilibrate into the flavin until NADH dissociates. Our rapid reaction experiments demonstrate that the increase in NADH fluorescence coincides with a redistribution of electrons in XDH according to the relative redox potentials of the various centers. Our interpretation is that NADH dissociation is a relatively slow step (~42 s$^{-1}$) accompanied by modest changes in the visible spectrum of XDH. Following this slower step, rapid electron redistribution causes the majority of the observed signal change. Dissociation of the bound ligand, NADH in this case, appears to be a trigger for intramolecular electron transfer. This property has been observed in the redox half-reactions of XDH (16) and XO (23) as well as in the reduction of other enzymes containing multiple redox centers (24, 25). In the current study, this characteristic occurs in the oxidative half-reaction. Observation of this phenomenon in both reductive and oxidative half-reactions supports control of intramolecular electron transfer by product dissociation as a general property of complex redox systems.

The reaction of chicken liver XDH with NAD has been studied at 4 °C, pH 7.8 (26). On mixing reduced chicken liver XDH with excess NAD a monophasic reaction was observed throughout the visible spectrum. The reaction demonstrated hyperbolic dependence of the observed rate constant on NAD concentration with a limiting rate constant of 27 s$^{-1}$ and an apparent $K_D$ of 90 μM. Unlike bovine milk XDH, reduction of NAD is the slow step, with NADH dissociation rapidly following. At the end point of the reaction, the enzyme appeared to be mostly XDH$_{4e}$. A model was presented in which two rounds of oxidation yield XDH$_{2e}$ and 2 eq NADH. Another difference between the two reactions is their end points. Bovine milk XDH is reduced by NADH to approximately XDH$_{3e}$ on average and is oxidized by NAD to approximately XDH$_{5e}$, consistent with simple oxidation-reduction equilibria. Chicken liver XDH reaches XDH$_{2e}$, whether it is reduced with NADH or oxidized with NAD, contrary to the predictions of a thermody-
namic equilibrium. This odd behavior was explained by the strong binding of NADH to the chicken liver enzyme, thus perturbing the equilibrium. The mammalian enzyme is thus less discriminate than the avian enzyme in regard to using the pyridine nucleotide as an oxidant or as a reductant.

It has previously been reported that XDH operates with a ping-pong mechanism during catalysis (16). A reasonably complete steady-state mechanism is presented in Scheme 2 in which XDH cycles between the 2- and 4-electron reduced states. It is certainly possible that other redox states are populated to a minor extent during turnover. These other oxidation levels are of little concern as reduction (16) and oxidation rates are mostly independent of the redox state of the enzyme. Oxidation of xanthine occurs at the molybdenum center (27), whereas reduction of NAD occurs at the flavin site (17, 16). Equilibration of electrons has been shown to be rapid with respect to \( k_{\text{eq}} \), consistent with the rapid equilibrium hypothesis of XDH catalysis (23). A steady-state rate equation was derived for the mechanism in Scheme 2 (see "Materials and Methods"). Product association was assumed to be zero under initial rate conditions. Reported rate constants were used for the reductive half-reaction (16). Note that values given for \( k_1 \), \( k_2 \), \( k_6 \), and \( k_{12} \) are lower limits that were estimated from simulations and a rapid binding equilibrium assumption. Values for the oxidative half-reaction were taken from Scheme 1. Using the mechanism and rate constants in Scheme 2, a \( k_\text{cat} \) of 5.5 s\(^{-1}\) was calculated. This agrees tolerably well with the experimental \( k_\text{cat} \) of 6.3 s\(^{-1}\) (4). \( K_m \) values for xanthine and NAD were calculated to be 1.1 and 2.2 \( \mu \)M, respectively. Experimentally determined \( K_m \) values are 0.3 \( \mu \)M for xanthine and 6.7 \( \mu \)M for NAD (4). These calculated \( K_m \) values agree within the error at which \( K_m \) values this low can be measured. The mechanism in Scheme 2 provides a reasonable model for XDH catalysis, although the enzyme need not function purely in a ping-pong fashion; the sites of oxidation and reduction are distant.

The reaction between XDH\(_{2e} \) and NAD is predicted to be a slightly unfavorable equilibrium, \( K_{\text{eq}} \) of 0.68. As this reaction has been implicated in catalysis, it is of some importance. The equilibrium between XDH\(_{2e} \) and NAD may be perturbed in the forward direction by the binding of NAD to free XDH\(_{2e} \), step \( k_{12}/k_{16} \) in Scheme 1. This sensitivity of the reaction end point to NAD binding is supported by simulations. Binding of NAD to XDH\(_{2e} \) could possibly be part of normal turnover. The sites of xanthine reduction and NAD oxidation are distant in XDH. Under some conditions, XDH may function by a ternary-complex mechanism in which xanthine oxidation and NAD reduction occur independently, with the 2Fe2S centers acting as an electron sink to maintain a store of reducing equivalents. Such ternary-complex behavior has been observed with XDH using 4-hydroxypyrimidine as the reducing substrate and NAD as the oxidizing substrate (29). The insensitivity of the oxidative half-reaction to ligands at the molybdenum center, including urate, supports the notion that oxidation at the flavin center can occur independently of chemistry at the molybdenum site.

If XDH cycles between XDH\(_{2e} \) and XDH\(_{4e} \) during xanthine/oxygen turnover, then XDH\(_{2e} \) is the species for which oxygen and NAD compete. The experiments described here demonstrate that XDH reacts with NAD rapidly and with high affinity. To explain the preference this enzyme form has for NAD over oxygen, the reaction of reduced XDH with oxygen must be considered. The fastest rate constant observed for the oxidation of bovine milk XDH with molecular oxygen is \( 7 \times 10^4 \) M\(^{-1}\) s\(^{-1}\) (5). This is 570-fold less than the estimated rate of NAD binding in Scheme 1, 4 \( \times \) 10\(^5\) M\(^{-1}\) s\(^{-1}\) (5). The rate constant of NAD reduction, 130 s\(^{-1}\), is still much greater than the fastest rate at which oxygen could react at air saturation, 18 s\(^{-1}\). The predicted capacity of NAD to out-compete oxygen as an XDH substrate is supported by the remarkably poor ability of oxygen to inhibit xanthine/NAD turnover (5). Thus NAD is the preferred electron acceptor for XDH due to its tight and rapid binding and its fast rate of reduction.

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