Rapid Reaction Studies on the Reduction and Oxidation of Chicken Liver Xanthine Dehydrogenase by the Xanthine/Urate and NAD/NADH Couples*

Lawrence M. Schopfer and Vincent Massey

From the Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

Takeshi Nishino

From the Department of Biochemistry, Yokohama City University School of Medicine, Yokohama 236, Japan

Chicken liver xanthine dehydrogenase can be partially reduced by either xanthine or NADH. Reduction to approximately the 2-electron-reduced level occurs with NADH, and reduction beyond the 2-electron level occurs with xanthine. In both cases, the reaction is triphasic. The first and third phases are dependent on reductant concentration, whereas the second phase is not. Oxidation of fully (6-electron) reduced xanthine dehydrogenase by either urate or NAD is monophasic and dependent on the oxidant concentration. Oxidation stops at about the same level of reduction that was reached by the corresponding reductant. The position of this end point is sensitive to the potential of the reactants but is relatively insensitive to excess concentrations of oxidant or reductant. NADH binding to 2-electron-reduced enzyme is implicated in fixing the end point position in those reactions involving pyridine nucleotides, whereas urate binding is involved in fixing the end point of those reactions involving xanthine and urate.

Xanthine dehydrogenase (XDH) catalyzes the oxidation of xanthine to uric acid with concomitant reduction of NAD to NADH. It is a dimer containing four oxidation-reduction active cofactors/monomers: one FAD, one molybdo-perin, and two 2Fe/2S centers of the spinach ferredoxin type. These centers provide the oxidant with the capacity for holding a maximum of six electrons, i.e. 3 eq of xanthine. In addition to xanthine, XDH is reducible by NADH (1).

Earlier titration work showed that whereas both xanthine and NADH are capable of reducing XDH (2), reduction was incomplete and appeared to stop at the 4-electron level. Stopped-flow studies on turkey liver XDH at 25 °C indicated that this reduction process was essentially monophasic, with both reductants (3). A second, slow phase was also noted and attributed to partial reduction of XDH to XDH*. These results were taken to indicate the existence of a thermodynamic barrier to further reduction which was imposed by the relative oxidation-reduction potentials of the reagents.

Using chicken liver XDH at 4 °C, we have re-examined the rapid reaction kinetics of reduction by these substrates. In addition, we have examined the rapid reactions involved in oxidation of XDH by NAD and urate. We find that rapid reduction (with either reductant) proceeds in three phases, the rates for two of which are dependent on reductant concentration. Oxidation (with either oxidant) occurs monophasically. The reaction stops at approximately the level of XDH* when either NADH is used to reduce XDH or NAD is used to oxidize XDH*. Similarly, the reaction stops at a point, intermediate between XDH* and XDH**, when either xanthine is used to reduce XDH or urate is used to oxidize XDH*. The end point is dependent on the potentials of the reactants and is insensitive to excess concentrations of substrate. In addition, there is evidence to suggest that a strong binding between NADH and XDH contributes to fixing the end point position in the reactions involving pyridine nucleotides. A similar role can be ascribed to urate in reactions involving xanthine and urate. Thus, there is a major role for ligand binding in defining the reactions of XDH.

EXPERIMENTAL PROCEDURES

Materials

Xanthine dehydrogenase (EC 1.1.1.204) was purified from livers of chickens fed on a high protein diet as previously described by Nishino (4) and stored on ice in the presence of 1.0 mM salicylate. An appropriate aliquot of stock XDH was passed over Sephadex G-25 equilibrated in 50 mm potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA immediately prior to each experiment. The XDH concentration was determined spectrophotometrically using an extinction coefficient for the oxidized enzyme of 37.0 mM cm⁻¹ at 456 nm (1). All XDH concentrations given in this work refer to the concentration of monomeric active sites.

β-NADH (Grade III) and β-NAD (Grade V) were purchased from Sigma. Commercial NAD was found to contain a contaminant which interacted with reduced XDH in a second-order fashion, causing a rise in absorbance at 450 nm, but no change at 340 nm. The rate of this reaction was faster than the NAD-dependent oxidation. This contaminant could be separated from NAD by passage over Sephadex G-10 (Pharmacia LKB Biotechnology Inc.) in water. Identification of the contaminant was not attempted. Gel filtration of commercial NAD has been found to remove an unidentified compound which was a reductant for XDH. No reductant was found in the samples of NAD used in these studies.

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1. The abbreviations used are: XDH, xanthine dehydrogenase, without consideration of its level of reduction: XDH**, oxidized xanthine dehydrogenase; XDH*, 2-electron-reduced xanthine dehydrogenase; XDH**, 4-electron-reduced xanthine dehydrogenase; XDH*, 6-electron-reduced xanthine dehydrogenase; 3-AcPy-NAD, 3-acetylpyridine-NAD; 3-AcPy-NADH, 3-acetylpyridine-NADH; AAD, 3-aminopyridine-adenine dinucleotide.

2. T. Nishino, unpublished results.
3-Acetylpyridine-NAD (3-AcPy-NAD) and 3-acetylpyridine-NADH (3-AcPy-NADH) were obtained from Pharmacia LKB Biotechnology Inc. Commercial 3-AcPy-NAD was also found to contain contaminants. Both a reductant and a rapidly binding compound were detected. Passage of 3-AcPy-NAD over Sephadex G-10 in a manner similar to that used for NAD reduced the size of the artifacts detected in the reactions, but did not eliminate them.

3-Aminopyridine-adenine dinucleotide (AAD) was synthesized by the method of Fisher et al. (5). As a precaution, AAD was also passed over Sephadex before use, but no evidence of contaminants was ever noticed.

Concentrations of the pyridine nucleotides were determined spectrophotometrically using established extinction coefficients: NADH at 340 nm, ε = 6.22 mM⁻¹ cm⁻¹ (6, 7); NAD at 259 nm, ε = 17.8 mM⁻¹ cm⁻¹ (7); 3-AcPy-NADH at 363 nm, ε = 9.1 mM⁻¹ cm⁻¹ (7); 3-AcPy-NAD at 260 nm, ε = 16.4 mM⁻¹ cm⁻¹ (7); and AAD at 331 nm, ε = 3.09 mM⁻¹ cm⁻¹ (5).

Sodium dithionite was a generous gift from Virginia Chemical Co. (Portsmouth, VA). Lumiflavin 3-acetate was a generous gift from Dr. S. Ghisla (University of Konstanz). The concentration of lumiflavin 3-acetate was determined using its extinction coefficient, ε = 12.5 mM⁻¹ cm⁻¹ at 448 nm. All other chemicals were of reagent-grade and were used without additional purification.

Methods

Absorbance spectra were recorded with either a Cary Model 17 or a Cary Model 219 double-beam spectrophotometer. Both sample and reference compartments were temperature-controlled and flushed with dry air to prevent condensation at low temperatures. All reactions were performed in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA at 4 °C, unless otherwise indicated.

Anaerobic Techniques—Anaerobic enzyme samples were prepared in an all-glass apparatus (8) by sequential evacuation and re-equilibration with oxygen-free nitrogen. Oxygen-free nitrogen was prepared by passing pre-purified nitrogen over a heated column of Riddox (Fischer) to remove traces of oxygen. Reagents were made anaerobic by flushing with oxygen-free nitrogen for 15 min in gas-tight Luer-tipped syringes.

Rapid Reactions—Rapid reaction absorbance measurements were made in a temperature-controlled, anaerobic stopped-flow apparatus which was interfaced to a Nova II (Data General) computer (9). A Watten 15 cutoff filter was placed in the light beam in front of the sample cell in order to reduce stray light for measurements at wavelengths greater than 530 nm. When used to take spectra, the stopped-flow instrument was programmed to scan at 60 nm/s.

Rates for the observed absorbance changes were determined from plots of ln([A]t - [A]∞)/(t - t0) versus time, where [A]t is the initial absorbance, [A]∞ is the final absorbance at each time point, and ln is the natural logarithm. For a first-order reaction, the plot was linear, and the slope of the line was equal to the rate constant.

Multiphasic reactions were treated as sequential first-order processes, i.e. a sum of exponential terms. The rate for the slowest phase was determined as described above. The contribution from this exponential was then subtracted from the original data, and the next phase was analyzed. This exponential stripping procedure is discussed by Fersht (10).

Activity Assay—The activity of XDH was routinely assayed at 25 °C in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA, 0.15 mM xanthine, and 0.5 mM NAD as described by Rajagopalan and Handler (1). The reaction was monitored by following the change in absorbance at 295 nm.

Analysis of Single-turnover Kinetic Patterns Showing Inverse Substrate Dependence—In 1966, Kirschner et al. (11) reported temperature-jump relaxation studies on D-glyceraldehyde-3-phosphate dehydrogenase in which they found that the rate of one step in the reaction decreased as the substrate concentration was increased. This phenomenon was attributed to a mechanism such as that shown in Scheme I.

\[
\frac{k_1}{k_2} = \frac{B}{C} = \frac{k_{2B}}{k_{2C}}
\]

SCHEME I

Subsequent observations of this type have been made for alkaline phosphatase (12) and α-chymotrypsin (13). In all instances, the rates k2B and k2C were taken to be large relative to the rates k1 and k-1.

Using the steady-state approach of Strickland et al. (14) and solving for the disappearance of species A, a general expression for the apparent rate of the mechanism in Scheme I can be derived.

\[
k_{app} = \frac{k_1}{k_2} \left( \frac{h_{k_1} X + h_{k_1} X + h_{k_2} X + h_{k_2} X}{h_{k_1} X + h_{k_2} X + h_{k_2} X} \right)
\]

If k2X and k-2 >> k1 and k-1, then the following is true.

\[
k_{app} = \frac{k_1}{k_2} \left( \frac{h_{k_1} X + h_{k_2} X + h_{k_2} X}{h_{k_2} X + h_{k_2} X + h_{k_2} X} \right)
\]

Comparable expressions have been presented by Halford (12) and Fersht and Yolanda (13). The limiting rate at high concentration of X is k1. Rearranging Equation 2, Equations 3 and 4 follow.

\[
k_{app} = \frac{k_1}{k_2} \left( \frac{h_{k_1} X + h_{k_2} X}{h_{k_2} X + h_{k_2} X} \right)
\]

(3)

\[
k_{app} = \frac{k_1}{k_2} \left( \frac{h_{k_1} X + h_{k_1} X}{h_{k_1} X + h_{k_1} X} \right)
\]

(4)

Plotting 1/(kapp - k1) versus the concentration of X gives a straight line with a non-zero y axis intercept. The intercept is equal to 1/k1. The slope of the line divided by the intercept gives k2/k-2, which is the reciprocal of the dissociation constant for the binding of X to species B. This analysis is predicated on the assumption that the ligand binding rates are fast relative to the preceding equilibrium rates.

RESULTS AND DISCUSSION

Reductive Titration with Dithionite—The visible absorbance spectrum of XDH was (Fig. 1) has maxima at 320 and 456 nm (ε = 37 mM⁻¹ cm⁻¹) with long wavelength absorbance extending beyond 800 nm. By analogy with xanthine oxidase, absorbance in the 450 nm region is approximately 50% due to flavin and 50% due to Fe/S, whereas absorbance in the 550 nm region is due only to Fe/S (15, 16). Three equivalents of dithionite (six electrons) result in maximal bleaching of the XDH spectrum. This differs from xanthine oxidase, where a dithionite-reducible disulfide takes up an additional equivalent of dithionite, making a total of 4 eq necessary for maximal bleaching.
bleaching of the chromophores (17). We have performed room temperature reductive titrations of XDH (using dithionite) in order to examine the response of the various reducible centers to stepwise reduction. It is implicit in the following discussion on the stages of XDH reduction that the various oxidation-reduction centers in the active site do not respond independently. As with xanthine oxidase, electron density is considered to be distributed throughout the active site, directed by the relative oxidation-reduction potentials of the centers (18). When we propose reduction of a specific group at some stage of reduction, we are referring to the group most significantly affected.

An anaerobic, room temperature titration of XDH** with dithionite revealed four stages in the course of reduction (Figs. 1 and 2). The first electron equivalent caused a pronounced decrease in absorbance at 450 nm, accompanied by a decrease of about 50% at 520 nm, with no increase in absorbance at longer wavelengths. This suggests reduction of one Fe/S center. The next 2 electron eq caused a marked increase in the 620 nm absorbance, accompanied by a further decrease at 450 nm and only a small change at 520 nm. The rise at 620 nm indicates that flavin neutral semiquinone (FADH*) was formed in this stage. By analogy with the neutral semiquinone of flavodoxin, one would expect a rise in absorbance at 520 nm equivalent to that seen at 620 nm upon formation of this radical (19). That the absorbance at 520 nm remains essentially unchanged argues that an offsetting loss in absorbance at 520 nm occurs at the same time. Such a loss in absorbance can only be attributed to reduction of the second Fe/S center. The extinction changes at 520 nm for reduction of Fe/S and formation of FADH* of nearly equal size and opposite direction (15, 19). Addition of 2 more electron eq results in a precipitous drop in absorbance at both 620 and 520 nm, indicating reduction of FADH*. As only one electron is involved in reduction of FADH*, molybdenum must also be reduced in this stage. Finally, there are minor losses in absorbance at all three wavelengths for the 6th electron eq, suggesting primarily reduction of molybdenum. These results imply the following relative order of oxidation-reduction potentials for the active site centers of XDH at 25 °C: Fe/S II > FAD/FADH* ≈ Fe/S I > Mo(VI)/Mo(V) > Mo(V)/Mo(IV). Note that this order is slightly different from that which we have inferred previously (20). It is also different from the sequence derived from the low temperature results of Barber et al. (21) which gives the order: Fe/S II > Fe/S I > Mo(V)/Mo(V) > FAD/FADH* > Mo(VI)/Mo(V) ≫ Mo(V)/Mo(IV). The discrepancy might reflect shifts in potential due to the large difference in temperature between our studies and those of Barber et al. (21). Temperature-dependent shifts in pH leading to shifts in the relative potentials of the xanthine oxidase centers have been documented (22). Use of the designations Fe/S I and Fe/S II is by analogy with the assignments of Barber et al. (21).

Alteration of the potentials in response to ligand binding is a recurring theme in flavoproteins (23, 24). We therefore sought to test this possibility with XDH by titrating an XDH**-AAD complex with dithionite. AAD is an NADH analog which does not undergo electron transfer (5).

Titration of the XDH**-AAD complex proceeded in three stages (Fig. 2B). The first electron reduction could not be separated from the second. Both caused a pronounced increase in absorbance at 620 nm, accompanied by a significant loss in absorbance at 520 nm. This suggests simultaneous reduction of Fe/S and formation of FADH*. Since the 520 nm extinction changes for Fe/S and flavin reduction are of opposite sign and nearly equal size, the net loss of absorbance at 520 nm argues that more Fe/S than flavin is reduced in this stage. Addition of the next electron equivalent involves minimal change in absorbance at 620 nm, with continued loss of absorbance at 450 and 520 nm. This suggests that formation of FADH* is matched by its further reduction to FADH2, whereas Fe/S continues to be reduced. The last 3 electron eq caused a continuous and nearly linear loss of the remaining absorbance at all wavelengths, indicating reduction of molybdenum and FADH* in an essentially parallel fashion. These results suggest the following alteration to the sequence of oxidation-reduction potentials for the components of XDH: Fe/S II ≈ FAD/FADH* > Fe/S I ≈ FADH*/FADH2 > Mo(VI)/Mo(V) ≫ Mo(V)/Mo(IV). The amount of FADH* can be estimated by using the 620 nm absorbance change and the extinction coefficient for neutral flavin semiquinone taken from *Megasphaera elsdensii* flavodoxin (3450 mol⁻¹ cm⁻¹ at 620 nm) (19). In the absence of AAD, a maximum absorbance change (initial) corresponding to 3.48 μM FADH* or 29% of the total XDH was measured. This compares to a maximum of 48% FADH* estimated by low temperature (80° K) EPR for a dithionite titration under similar conditions (21). In the presence of AAD, the maximal 620 nm absorbance change corresponded to 4.35 μM FADH*, or 61% of the total XDH. The major effect of AAD binding to XDH** thus seems to shift the potential of the FAD/FADH* couple toward that of Fe/S II, causing a greater separation from the FADH*/FADH2 couple, with a consequent increase in the maximal fraction of FADH* observed.

Reduction of XDH by NADH—Although NADH is a product in the physiological reaction, it can efficiently reduce XDH** (1). In order to understand better the pyridine nucleotide mechanisms, we have examined its reaction with XDH** in the stopped-flow spectrophotometer.

An aerobically mixing XDH** with NADH (4 °C) resulted in a triphasic reaction leading to the partial reduction of XDH (Figs. 3 and 4). The fast phase caused a rise in absorbance at 620 nm (first part of a biphasic rise) and a fall at 550 and 450 nm, suggesting the formation of FADH* and reduced Fe/S II. This combination of reduced centers as the first observable

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**Fig. 2. Plots of absorbance versus electron equivalents added for dithionite titrations of oxidized xanthine dehydrogenase plus and minus AAD. A, conditions as were those described for Fig. 1; B, XDH** (7.1 μM) in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA and 0.3 mM AAD at 25 °C was made anaerobic and titrated with sodium dithionite (2.8 mM). Dithionite was standardized using lumiflavin 3-acetate, as described for Fig. 1. The points in each panel are measured absorbance values from spectra taken after each addition of dithionite: •, 520 nm; □, 620 nm; △, 550 nm; ▲, 450 nm. Selected points in A are shown as full spectra in Fig. 1. The number of electron equivalents was calculated from the volume of dithionite added.
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FAD to give FADH$_2$ would be expected. It follows that the rate of redistribution of electrons from FADH$_2$ to Fe/S II, with the concomitant formation of FADH$^+$, must be faster than the fastest observed reduction rate (160 s$^{-1}$; see below). The observed rate can therefore be assigned to the rate of electron transfer between NADH and FAD to form FADH$_2$.

The thermodynamic driving force for the subsequent redistribution of electrons can be attributed to the high redox potential of Fe/S II. That the rate of redistribution is faster than the rate for electron transfer from NADH to XDH is consistent with the proposals of Olson et al. (18, 27) for milk xanthine oxidase.

The observed rate for this phase showed a saturating dependence on NADH concentration, indicating the existence of a Michaelis-Menten complex between NADH and XDH$^{**}$ prior to electron transfer. The measured rate for this phase was consistently different when measured at different wavelengths (620 > 450 > 550 nm), a feature also noted with the turkey liver enzyme (3). Plotting the data for each wavelength in double-reciprocal fashion (1/[$k_{\text{cat}}$ versus 1/[NADH]) (14) gave straight lines with non-zero y axis intercepts (Fig. 5A).

An apparent dissociation constant of about 3.5–7.3 μM for the formation of the XDH$^{**}$-NADH complex and limiting rates of 160 s$^{-1}$ (620 nm), 106 s$^{-1}$ (450 nm), and 84 s$^{-1}$ (550 nm) were found. The scatter in the data at 550 and 620 nm reflects the small size of this phase at those wavelengths. The amount of FADH$^*$ formed in this phase can be estimated from the 620 nm absorbance change. About 25–30% of the flavin is in the FADH$^*$ form at the end of this phase. The reason for the wavelength dependence of the rate of this phase is still unclear. It is apparent, however, that the fastest event involves formation of FADH$^*$. Since two electrons are initially associated with the flavin, formation of FADH$^*$ requires only that one electron be transferred to another center. Transferring an electron from FADH$_2$ to molybdenum would result in a rise at both 550 and 620 nm due to the formation of FADH$^*$ as discussed above. Transferring an electron to Fe/S II would result in a rise at 620 nm and a lag at 550 nm. The observed loss in absorbance at 550 nm together with the 25–30% yield in FADH$^*$ argues that electron density is transferred from FADH$^*$ to other centers (probably Fe/S) at the same time that it is being transferred from FADH$_2$. These processes are not well resolved, although the fact that FADH$^*$ accumulates would argue that it is formed at approximately the same rate as it decays. This description of the events engenders FADH$^*$

Fig. 3. Plots of absorbance versus time for reduction of oxidized xanthine dehydrogenase by NADH. XDH$^{**}$ (7.8 μM) in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA at 4 °C was anaerobically mixed with an equal volume of NADH (100 μM) in the same buffer using a stopped-flow spectrophotometer (2-cm optical path length). Absorbance changes, at the wavelengths indicated in each panel, are shown for time periods of 80 ms (---) and 0.8 s (— — —).

Fig. 4. Spectra for reaction of NADH with XDH$^{**}$ and NAD with XDH$^{**}$ under various conditions. Spectra were taken with a 2-cm optical path length observation cell in a stopped-flow spectrophotometer. For lines 1 and 2, conditions were as described for Fig. 3 (normalized to 4.4 μM XDH). Line 1, spectrum of XDH$^{**}$ line 2, final spectrum of partially reduced XDH after reaction with 36 μM NADH. For lines 3 and 4, conditions were as described for Fig. 6. Line 3, spectrum of XDH$^{**}$ (4.4 μM); line 4, final spectrum of partially oxidized XDH after reaction with 200 μM NAD. Inset, secondary plot of the absorbance at 450 nm versus the concentration of NAD, taken from an anaerobic titration of fully reduced XDH by NAD. XDH$^{**}$ (11.8 μM) was made anaerobic in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA and reduced with sodium dithionite. The resultant XDH$^{**}$ was anaerobically titrated with purified NAD (13.4 mM) at 4 °C. The optical density changes were complete within 1 min of mixing.

reaction product merits some comment. The FAD center of XDH is generally viewed as the site at which NADH reacts (16). Since reaction of NADH with FAD occurs two electrons at a time (25, 26), an obligatory two-electron reduction of

Fig. 5. Secondary plots of rate dependence on NADH concentration from reaction of XDH$^{**}$ with NADH. Conditions were the same as described for Fig. 3. A, double-reciprocal plot of the fast phase rate at 550 nm (■), 450 nm (○), and 620 nm (▲). B, plot of the reciprocal of the observed rate (1/k$_{\text{cat}}$) versus the concentration of NADH minus 1 eq of XDH. This analysis is described under "Experimental Procedures." The NADH concentration has been reduced by 1 eq of XDH to accommodate the NADH utilized in the fast phase.
with an intermediary, steady-state function. Thus, the absorbance changes at 620 nm would be expected to stop sooner than those at 550 nm. Such a sequence of events might rationalize the wavelength dependence of the observed rates for this phase. The very existence of a wavelength dependence in the observed rates indicate that this phase represents a complicated process.

The second phase was seen most distinctly at concentrations of NADH greater than 80 μM and was made clear by the exponential stripping procedures employed. It appeared as a lag or small rise in absorbance at 550 nm and as a continued rise in absorbance at 620 nm (Fig. 3). The rise at 620 nm was consistently 2–3-fold larger than that at 550 nm. As the extinction coefficients for flavin semiquinone are typically approximately equal at 620 and 550 nm, we can infer that formation of FADH* is accompanied by reduction of Fe/S in this phase. The maximum rise in 620 nm absorbance corresponded to about 65% of the FAD being present as FADH*. The apparent rate of this phase appeared to be independent of NADH concentration, with a value of about 25 s⁻¹. The absence of an NADH concentration dependence suggests that the observed redistribution of electron density may be triggered by release of NAD from the enzyme. However, the dithionite titrations of Fig. 2 suggest that the pyridine nucleotide-complexed form of XDH* should stabilize FADH*. Release of NAD would then be expected to cause a decrease in the FADH* concentration, not an increase as is observed in this phase. In order to rationalize our interpretation with the titration observations, we must propose that binding NAD to XDHz has the opposite effect of binding AAD to XDHz*. At 450 nm, there is also an NADH-independent second phase. However, it occurs at a rate of about 10 s⁻¹. Unlike the situation at 550 and 620 nm, there is no evidence at 450 nm for a third phase in the reduction. The slower measured rate at 450 nm may reflect a combination of overlapping events which are incompletely resolved at this wavelength. The middle phase at 550 and 620 nm becomes resolved only at high NADH concentrations. This is a consequence of the NADH-dependent increase in the rate of the first phase and a corresponding decrease in the rate of the third phase.

The third phase resulted in loss of absorbance at 550 and 620 nm. The apparent rate for this phase showed an inverse dependence on NADH concentration, the measured rate becoming smaller as the concentration of NADH was increased. Although this is an unusual substrate dependence, it is not unprecedented (11–13). The simplest model for a substrate dependence of this sort consists of a rate-limiting equilibration between two forms of the enzyme, with a ligand binding to only one form (see Scheme I). Analysis of this sort of mechanism is described under "Methods." The limiting rate at high NADH concentrations approached 1.9 s⁻¹, which represents the forward rate of equilibration  (kₐ in Scheme II). Plotting 1/(kₛᵤₛ + kₑ) versus [NAD - XDH] gave a straight line with a non-zero y axis intercept (Fig. 5B). The reciprocal of the intercept gave a value of 16 s⁻¹, which is the reverse rate of the equilibrium (kₑ in Scheme II). A value of 11 μM for the apparent dissociation constant of the enzyme-NAD complex was obtained from the reciprocal of the slope of Fig. 5B divided by the intercept.

A second NADH-dependent rate would normally indicate the point of entry for a second pair of electrons. However, from the final absorbance at 450 nm, only approximately 2 electron eq appear to have been taken up by XDH. Fifty-seven percent of the absorbance at 450 nm was bleached (XDH** - XDH* = 100%) by 18 μM NADH (Fig. 4). This represents the end point of reduction. Increasing the NADH concentration up to 180 μM caused only 7% additional bleaching. The dithionite titrations (Fig. 2, A and B) indicate that 51–53% bleaching at 450 nm occurs upon 2-electron reduction and 89–91% upon 4-electron reduction. This argues that the third phase does not represent any substantial reduction of XDH**, despite the dependence on NADH concentration. This in turn argues that the observed loss of absorbance at 620 and 550 nm reflects a redistribution of electron density in response to NADH binding to XDHz. NADH binding must therefore shift the flavin potentials to destabilize FADH*. This is contrary to the observations with AAD, but consistent with our proposal for the effect of NAD.

The above data on reduction of XDH** by NADH and our interpretations are summarized in Scheme II. It is important to emphasize that the data clearly indicate that an overall equilibrium is established, so that some of all of the species will be present, as well as a small percentage of the enzyme in the four-electron-reduced state.

Oxidation of XDH** by NAD—If the reduction of XDH** by NAD proceeds to an approximately 2-electron-reduced equilibrium end point, then oxidation of XDH** by NAD might be expected to approach the same final state.

Anaerobically mixing NAD with dithionite-reduced XDH resulted in a monophasic increase in absorbance at all wavelengths examined: 340, 450, 550, and 650 nm. This increase in absorbance amounted to only partial return of the absorbance of oxidized enzyme at 450 and 550 nm (Fig. 4, line 4), suggesting that only partial oxidation had occurred. The rate for this reaction showed a saturating dependence on NAD concentration. A plot of the reciprocal of the apparent rate (1/kₛᵤₛ versus the reciprocal of the NAD concentration (1/[NAD]) was linear for NAD concentrations less than 50 μM (Fig. 6). Analysis (14) of the double-reciprocal plot gave a limiting rate of 27 s⁻¹ and an apparent dissociation constant for the XDH**-NAD complex of 80 μM. At higher NAD concentrations, the oxidation rate was less than that predicted from the lower NAD measurements, suggesting excess NAD inhibition (Fig. 6).

The spectrum of the final reaction mixture at 100 μM NAD (Fig. 4, line 4) was nearly identical to that found for the end point of the NADH/XDH** reaction (Fig. 4, line 3). The end point of the oxidation increased as the NAD concentration increased from 12.5 to 100 μM; but from 100 to 200 μM, it remained essentially unchanged. At higher NAD concentrations, XDH became only slightly more oxidized. This latter finding is consistent with the results of titrations in which dithionite-reduced XDH was anaerobically mixed with NAD (Fig. 4, inset). Addition of NAD caused a partial oxidation of XDH, which was essentially complete by 100 μM. However, subsequent addition of NAD up to 1.9 mM caused a small extent of further oxidation. We suggest that this biphasic titration indicates oxidation of XDH** to an equilibrium, approximately 2-electron-reduced level, followed by the binding of NAD to XDHz, causing a shift in the equilibrium toward XDH**. Binding of NAD to XDH** was previously indicated by the protection against chemical modification which NAD provides to XDHz (28). Thus, both reactions NADH/XDH** and NAD/XDH** reach the same equilibrium end point.

The finding that the end point is essentially independent of either excess NADH or excess NAD is inconsistent with a simple oxidation-reduction equilibrium and implies that the equilibrium is strongly influenced by the binding of either NAD or NADH. In view of the apparent influence of NADH on the potentials of XDH** described above, we suggest that NADH is the operative ligand.
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**Scheme II.** The structures depicted in each step represent our choice for the major resonance form. A distribution of electron density among the groups of the active site, based on the oxidation-reduction potentials of each group, more accurately describes the situation, but a scheme of that complexity would be awkward. The species shown in brackets are implicated in the mechanism, but not spectrally visible in the course of the reaction.

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**Fig. 6.** Secondary plot of $1/k_{app}$ versus $1/[NAD]$ for oxidation of fully reduced xanthine dehydrogenase by NAD. XDH$^{ox}$ (8.8 μM) in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA was made anaerobic and titrated to full reduction (six electrons) with sodium dithionite. XDH$^{ox}$ was then mixed anaerobically (4 °C) with an equal volume of purified NAD (25 μM to 1.2 mM) in the same buffer using a stopped-flow spectrophotometer (2-cm optical path length). XDH used in this experiment was 81% active. The reaction was monophasic at 340, 450, and 550 nm. The points are an average of three to four rate measurements taken at this set of wavelengths.

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Oxidation of XDH$^{ox}$ by NAD is summarized in Scheme III.

**Reaction of XDH with 3-AcPy-NAD/3-AcPy-NADH**—When XDH$^{ox}$ (dithionite-reduced) was anaerobically mixed with 3-AcPy-NAD (36 μM), greater than 85% return of the 450 nm absorbance occurred (Fig. 7, ---). The oxidation-reduction potential of the 3-AcPy-NAD/3-AcPy-NADH couple (−248 to −260 mV) (29, 30) is 60–72 mV more positive than that for the NAD/NADH couple (−320 mV) (31), making 3-AcPy-NAD more readily reducible than NAD. Thus, the increased oxidation of XDH is in accord with expectation. As the concentration of 3-AcPy-NADH was increased to 108 μM, progressively more 450 nm absorbance appeared, reaching an apparent limit at about 95% of the total. Increasing the 3-AcPy-NAD concentration to 525 μM gave no additional change. Oxidation appeared to be monophasic, with the apparent rate showing a saturating dependence on 3-AcPy-NAD concentration. A double-reciprocal plot ($1/k_{app}$ versus $1/[3-AcPy-NAD]$) gave values of 11 s$^{-1}$ for the limiting rate and 150 μM for the apparent dissociation constant. A slower, additional increase in absorbance at 340 nm, beyond that consistent with the extent of oxidation seen at 450 nm, indicated the presence of a contaminant capable of reducing XDH. Unlike NAD, passage of 3-AcPy-NAD over Sephadex G-10 did not eliminate this factor. This behavior is qualitatively similar to that described for NAD.

Anaerobic reduction of XDH$^{ox}$ with 15 μM 3-AcPy-NADH caused only about 18% bleaching of the 450 nm absorbance (Fig. 7, ---). The end point was comparable to that obtained by oxidation of XDH$^{ox}$ using low levels of 3-AcPy-NAD. Increasing the 3-AcPy-NADH concentration from 15 to 56 μM caused no change in this end point. As with NADH, reduction of XDH by 3-AcPy-NADH was multiphasic. At least two minor phases preceded the bleaching of absorbance at 450 nm. The 450 nm bleaching occurred with an apparent rate of about 1.5 s$^{-1}$, which was independent of 3-AcPy-NADH concentration. The faster phases were too small for accurate rate analysis. Thus, the overall behavior of the 3-AcPy-NAD/3-AcPy-NADH couple with XDH$^{ox}$/XDH$^{ox}$ is qualitatively the same as that seen with the NAD/NADH couple, except that the end point appears at a more oxidized level of XDH in the former case.

**Reduction of XDH$^{ox}$ by Xanthine**—Anaerobically mixing...
Oxidation-Reduction of Xanthine Dehydrogenase

Scheme III. As in Scheme II, the structures depict the major resonance form at each step. Bracketed structures are implicated in the mechanism but are not spectrally visible in the course of the reaction.

Fig. 7. Spectra taken from reaction of 3-AcPy-NAD with XDH\(^{++}\) and 3-AcPy-NADH with XDH\(^{--}\). Anaerobic XDH\(^{++}\) (8.8 \(\mu\)M) was titrated to full reduction with sodium dithionite. The resultant XDH\(^{--}\) was mixed (4°C) with an equal volume of purified 3-AcPy-NAD (36 \(\mu\)M) in the same buffer using a stopped-flow spectrophotometer. - - , spectrum of XDH\(^{++}\) (4.4 \(\mu\)M); ------, spectrum of XDH\(^{--}\) (4.4 \(\mu\)M); ----, final spectrum of partially oxidized XDH after the reaction. XDH\(^{++}\) (11 \(\mu\)M) was anaerobically mixed (4°C) with an equal volume of 3-AcPy-NADH (30 \(\mu\)M) in a stopped-flow spectrophotometer. - - - , spectrum of partially reduced XDH (normalized to 4.4 \(\mu\)M). All spectra presented were taken with a stopped-flow spectrophotometer (2-cm optical pathlength).

XDH\(^{++}\) with xanthine resulted in a tetraphasic reaction. The time courses of the three fastest phases are shown in Fig. 8. The four phases are indicated by the spectral changes in Fig. 9 (the solid triangle spectrum to the spectrum of line 3). The observed rate for this final phase was independent of xanthine concentration, with an apparent value of about 0.006 s\(^{-1}\). This phase led to a reappearance of absorbance at 620 nm, as can be seen in the 80-s trace of the 620 nm panel in Fig. 8. We propose that this final phase represents the transfer of reducing equivalents from active, reduced XDH to the inactive, oxidized desulfo form, a phenomenon well documented with both xanthine oxidase (32) and xanthine dehydrogenase (2, 33). As such, this phase is not a part of the normal, catalytic mechanism for XDH reduction by xanthine.

The three faster phases do, however, appear to be involved in the xanthine-mediated reduction of XDH. These three phases account for the majority of the spectral change observed, and the apparent rates are rapid enough to accommodate the measured steady-state turnover (see below). Our proposed mechanism for this reaction is summarized in Scheme IV.

Fig. 8. Plots of absorbance versus time at selected wavelengths for reduction of xanthine dehydrogenase by xanthine. XDH\(^{++}\) (9.8 \(\mu\)M) in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA was mixed anaerobically (4°C) with an equal volume of xanthine (50–400 \(\mu\)M) in the same buffer using a stopped-flow spectrophotometer (2-cm optical path length). The reaction time courses are shown in three time scales: 0.8 s (---), 16 s (-- --), and 80 s (----). XDH used for this experiment was 84% active.
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SCHEME IV. The structures depict the major resonance form at each step. Bracketed structures are implicated in the mechanism but are not spectrally visible. The brackets around urate (U) in the last structure represent ambiguity about its presence at that point in the reaction. X, xanthine.

Compounds were shown to interact with the molybdenum site of fully reduced xanthine oxidase, causing spectral perturbations similar to those observed in the fast phase of our studies (Fig. 9). On the basis of this similarity, we suggest that the fast phase represents 2-electron reduction of the molybdenum center to form an XDHuranate complex. The apparent rate \( k_{app} \) showed a saturating dependence on xanthine concentration. A double-reciprocal analysis (Fig. 10A) \( (14) \) yielded a limiting rate of 180 \( \text{s}^{-1} \) and an apparent dissociation constant for the xanthine-\( \text{XDH}^{-} \) Michaelis-Menten complex of 280 \( \mu \text{M} \). Lack of a major absorbance change in this phase indicates that neither flavin nor Fe/S was reduced.

The second phase (Fig. 9, M) resulted in a general decrease in absorbance from 320 to 575 nm, whereas at wavelengths greater than 575 nm, there was an increase. This phase lasted for about 2 s (Fig. 8, 620 nm panel). The apparent rate \( k_{app} \) was independent of xanthine concentration \( (10-200 \mu \text{M}) \). The absorbance rise in the 620 nm region corresponds to 30-40% of the total FAD becoming FADH\(^{+}\), whereas the fall in absorbance around 550 nm suggests reduction of an Fe/S center. If two electrons were associated with the molybdenum at the end of the first phase, the absorbance changes in the second phase indicate redistribution of electron density. Urate binding to XDH is known to shift the molybdenum potentials to more positive values, making molybdenum the most readily reducible center in the enzyme \( (35) \). The end point of this phase was unchanged for xanthine concentrations from 20 to 200 \( \mu \text{M} \). The apparent rate \( k_{app} \) showed a saturating dependence on xanthine concentration. A plot of \( 1/k_{app} \) versus \( 1/[\text{xanthine} - \text{XDH}] \) was linear, with a non-zero y axis intercept (Fig. 10B). Analysis of this double-reciprocal plot \( (14) \) gave a limiting rate of 0.77 \( \text{s}^{-1} \) and an apparent dissociation constant of 55 \( \mu \text{M} \) for xanthine. If our analysis of the preceding phases is correct, the starting point for this reaction is 2-electron-reduced XDH. The dependence of \( k_{app} \) on xanthine concentration together with the marked loss of absorbance at 450 nm argues that a second reduction step occurs in this phase. In fact, there was a total of 72% bleaching at 450 nm (allowing for 17% desulfoenzyme in this preparation) at the end of this phase. Comparing this value to the dithionite titrations (Fig. 2), we find that more than two, but less than four, electrons have been introduced. Thus, only a portion of the XDH is reduced to XDH\(^{4-} \). The simplest interpretation of the limiting rate for this phase \( (0.77 \text{ s}^{-1}) \) would be to assign it to the rate of electron transfer between xanthine and XDH\(^{+} \). This would, however, represent a 200-fold reduction in the rate of this step from that seen for XDH\(^{+} \). There is no independent evidence to

(Fig. 11). A rapid rise at 360 nm was consistent with formation of XDH\(^{-} \)urate. The subsequent loss of absorbance was comparable to that seen in the second phase with excess xanthine. The third and fourth phases were not seen. The final spectrum was quite similar to that of Fig. 9 (solid square spectrum), including the increased absorbance in the 600-650 nm region. Thus, the first two phases can be attributed to changes resulting from the addition of only two electrons to XDH, supporting the suggestion that the second phase occurs in response to urate release.

The third phase (Fig. 9, A) resulted in loss of absorbance at all wavelengths. This phase therefore involves reduction of FADH\(^{+} \) to FADH\(^{2-} \) (loss of 620 nm absorbance) and reduction of the second Fe/S center (additional loss of 550 nm absorbance). The end point of this phase was unchanged for xanthine concentrations from 20 to 200 \( \mu \text{M} \). The apparent rate \( k_{app} \) showed a saturating dependence on xanthine concentration. A plot of \( 1/k_{app} \) versus \( 1/[\text{xanthine} - \text{XDH}] \) was linear, with a non-zero y axis intercept (Fig. 10B). Analysis of this double-reciprocal plot \( (14) \) gave a limiting rate of 0.77 \( \text{s}^{-1} \) and an apparent dissociation constant of 55 \( \mu \text{M} \) for xanthine. If our analysis of the preceding phases is correct, the starting point for this reaction is 2-electron-reduced XDH. The dependence of \( k_{app} \) on xanthine concentration together with the marked loss of absorbance at 450 nm argues that a second reduction step occurs in this phase. In fact, there was a total of 72% bleaching at 450 nm (allowing for 17% desulfoenzyme in this preparation) at the end of this phase. Comparing this value to the dithionite titrations (Fig. 2), we find that more than two, but less than four, electrons have been introduced. Thus, only a portion of the XDH is reduced to XDH\(^{4-} \). The simplest interpretation of the limiting rate for this phase \( (0.77 \text{ s}^{-1}) \) would be to assign it to the rate of electron transfer between xanthine and XDH\(^{+} \). This would, however, represent a 200-fold reduction in the rate of this step from that seen for XDH\(^{+} \). There is no independent evidence to
suggest that the rate of electron transfer between xanthine and molybdenum should be affected by the state of reduction of either the Fe/S or flavin centers. Results from xanthine oxidase (18) in fact indicate that the intrinsic electron transfer rate is the same at all levels of reduction. A rationalization of this phenomenon is given in Scheme IV.

The reduction data provide us with direct evidence for the reaction of 2 equ molar xanthine with XDH. We have no evidence for the reaction of 2 eq of xanthine and urate. In the stopped-flow spectrophotometer experiments, the absorbance change was dependent on the concentration of urate, but the rate remained constant at all concentrations of urate (up to 1 mM). Oxidative reduction of XDH ended at a partially reduced level which was insensitive to either excess reductant (xanthine) or oxidant (urate). This also applies for the reaction of 2 eq of xanthine with XDH.

This interpretation gives a rate for urate release from XDH° (0.23 s⁻¹) which is 5-fold slower than the rate for urate release from XDH° (1.8 s⁻¹).

The extent of reoxidation appears to reach a limit at about 30 μM urate. As was seen in the oxidation by NAD, higher concentrations of urate (up to 1 mM) led to further, small amounts of oxidation. The XDH reduction level at 30 μM urate (Fig. 9, line 4) was about the same as that seen at the end of the fast phases during reduction of XDH° by xanthine. Under the conditions normally used for the XDH°/urate reduction experiments, about 10 μM urate would be formed. This amount of urate would be sufficient to prevent full reduction of XDH. Thus, we again have a situation in which oxidation-reduction of XDH ends at a partially reduced level which is insensitive to either excess reductant (xanthine) or oxidant (urate).
oxidant (urate). In this instance, the reduction appears to stop between the 2- and 4-electron levels. Strong urate binding appears to limit further reaction. Urate has previously been shown to serve as an electron acceptor in anaerobic turnover, with hypoxanthine as a substrate (38).

**Steady-state Turnover of XDH with Xanthine and NAD—**

From the foregoing rapid reaction studies, one would predict that XDH turnover occurs between 2- and 4-electron levels. At 4°C, initial rate turnover experiments were saturated in both xanthine (8-10 μM) and NAD (10-100 μM) (i.e., Km values too low to measure), giving a turnover number of 1.7 s-1. The turnover rate is equal to the rate for the second step in the xanthine reduction reaction, suggesting that urate release is rate-limiting in turnover. This is in accord with the faster rate found for oxidation of XDH by NAD (27°C) (39). Additional information on the status of XDH during steady state was obtained by using enzyme-monitored turnover (40) under anaerobic conditions. With 3.9 μM XDH and either 50 μM xanthine and 250 μM NAD or 250 μM xanthine and 545 μM NAD, we found that XDH was reduced to approximately the 2-electron level (50% bleaching at 450 nm) during approach to steady state, remained at that level throughout steady state, and was unchanged when the reaction was complete. The XDH steady-state spectrum is essentially identical to the end point spectrum obtained upon reducing XDH with NADH (Fig. 4), including the absence of 620 nm absorbance of FADH2 normally expected for XDH-. This indicates that during steady state, pyridine nucleotides remain bound to the XDH2- form. Since there was no change in the XDH spectrum on going from steady state to the end of the reaction, we could not use this method to evaluate the turnover parameters. However, the XDH steady-state spectrum at steady state in conjunction with the limiting rates for reduction by xanthine (1.8 s-1) and oxidation by NAD (27°C) argue that turnover occurs between 2- and 4-electron-reduced XDH, i.e., turnover consists of a rate-limiting reduction of XDH2- by xanthine to yield XDH4-, which is followed by a rapid, NAD-mediated oxidation back to XDH2-.

In Scheme IV, we have outlined our view of the sequence of events involved in reduction of XDH by xanthine and the oxidation of XDH2- by urate. The measured rate of 0.77 s-1 has been assigned to release of urate from XDH2-. This rate is too slow to be included in turnover. We therefore suggest that in turnover, NAD reacts rapidly with the XDH2-urate complex to give XDH4--urate, which then releases urate at the limiting rate of 1.8 s-1.

**Conclusions**

We have found that reduction of chicken liver xanthine dehydrogenase at 4°C in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA either by xanthine or NADH proceeds triphasischally (Figs. 3 and 8). The first and third phases are dependent on reductant concentration (Figs. 5 and 10). The middle phase is independent of reductant concentration and can be related to product release. The spectrum of the enzyme at the end of reduction by NADH is very similar to that of 2-electron-reduced XDH (Fig. 4), whereas that at the end of reduction by xanthine is closer to 4-electron-reduced XDH (Fig. 9). Oxidation of XDH2- with either urate or NAD proceeds in a single, oxidant-dependent phase to essentially the same level of reduction reached by the corresponding reductants (Figs. 4 and 9). In both cases, the end point position is insensitive to excess concentrations of oxidant and reductant.

Fhaolain and Coughlan (3) have presented results for the reduction of turkey liver xanthine dehydrogenase using the same buffer and pH, but at 25°C. They found two phases for reduction by either xanthine or NADH. The fast phase was dependent on reductant concentration, whereas the slow phase was not. The fast phase was fast enough to accommodate turnover, whereas the slow phase was not. These observations together with information from the literature that XDH could be readily titrated to about XDH2- by these reductants led them to conclude that the reductive half-reaction proceeded to 4-electron-reduced XDH in the fast phase and then onto 6-electron-reduced XDH (partially) in the slow phase. Due to the difficulties in reducing XDH beyond the 4-electron level, Fhaolain and Coughlan suggested that turnover must occur between XDH2- and XDH4-.

The details of our results are somewhat different. Some of the discrepancy can be attributed to the general tendency to resolve more phases at the lower temperature, whereas some may be due to the different enzyme sources. The general features of the two studies are, however, the same. Neither reductant is capable of fully reducing XDH, and the catalytic reaction cycles mainly between XDH2- and XDH4- We have further established that binding of urate and pyridine nucleotides play major roles in defining the reactivity of XDH.

Based on steady-state kinetic studies, Fenoll et al. (41) have also suggested that pyridine nucleotide can modify the reactivity of xanthine dehydrogenase.

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