Role of the Flavin Midpoint Potential and NAD Binding in Determining NAD Versus Oxygen Reactivity of Xanthine Oxidoreductase*

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Xanthine oxidoreductase from bovine milk can be prepared in two interconvertible forms, xanthine oxidase (XO) and xanthine dehydrogenase (XDH), depending on the number of protein cysteines versus cystines. Enzyme forms differ in their oxidizing substrates; XDH prefers NAD to molecular oxygen, whereas XO only reacts significantly with oxygen. The preference for oxidizing substrate is partially explained by thermodynamics. The oxidoreductase is too high in XO to efficiently reduce NAD (Hunt, J., Massey, V., Dunham, W.R., and Sands, R.H. (1993) J. Biol. Chem. 268, 18685–18691). To distinguish between changes in thermodynamics and in substrate binding, samples of both XO and XDH have been prepared in which the native NAD has been replaced with an FAD analog of different redox potential, 1-deaza-FAD or 8-CN-FAD. Reductive titrations indicate that both 1-deaza-XO and 1-deaza-XDH have a flavin midpoint potential similar to native XDH and that 8-CN-XO and 8-CN-XDH each have a flavin potential higher than XO. Both the low potential 1-deaza-XO and the high potential 8-CN-XDH contain essentially no xanthine/NAD activity. However, 1-deaza-XDH does exhibit xanthine/NAD activity, and 8-CN-XO has normal xanthine/oxygen activity.

The binding of NAD to oxidized XO and XDH was investigated by ultrafiltration and isothermal titration calorimetry. The $K_a$ for the binding of NAD to XO was determined to be $280 \pm 145 \mu M$ by ultrafiltration and $160 \pm 40 \mu M$ by isothermal titration calorimetry. No evidence for the binding of NAD to XO by either method could be obtained. A low flavin midpoint potential is necessary but not sufficient for dehydrogenase activity.

Xanthine oxidoreductase catalyzes the oxidation of hypoxanthine and xanthine to urate and is involved in purine catabolism in mammals. Isolated from bovine milk, the enzyme exists as a dimer, containing one molybdopterin, one FAD, and two plant ferredoxin-type 2Fe/2S centers per 145-kDa subunit (1–4). Reducing substrates such as xanthine react at the molybdenum center (5), whereas oxidizing substrates such as oxygen or NAD react at the FAD (6, 7). Like other molybdenum hydroxylases, the oxygen incorporated into substrate is derived from water, and electron equivalents are released in the hydroxylase reaction. Electrons from xanthine are transferred either to NAD or to molecular oxygen, depending on the form of the enzyme present. Xanthine dehydrogenase-type (XDH)1 enzyme prefers NAD as an electron acceptor (8) but in the absence of NAD will catalyze xanthine/oxygen turnover at 30% the rate of xanthine/NAD turnover (9). Xanthine oxidase-type (XO) enzyme only utilizes oxygen as its oxidizing substrate, to any significant extent. XDH from bovine milk can be converted to XO irreversibly by proteolysis (10, 11) or reversibly by oxidation of cysteines to cystines (8, 12). Approximately eight cysteines are oxidized to four cystines on converting milk XDH to XO (12). XDH is thought to be the major form present in vivo (13), and there is evidence that reduced oxygen species formed from XO or XDH may be important in several oxidative pathologies (14–16).

There is strong evidence for a conformational change between XDH and XO in the FAD binding region. XDH strongly destabilizes anionic forms of the flavin relative to XO (17, 18). XDH contains an NAD-binding site adjacent to the FAD, as addition of the NAD analog aminopyridine adenine dinucleotide causes marked perturbations of the visible spectrum of XDH (19, 20), and NAD causes only very small perturbations to XDH, $\Delta \epsilon = 600 \text{ m}^{-1} \text{ cm}^{-1}$. In contrast, no spectral changes occur on the addition of either compound to oxidized XO. The redox potential of the FAD/FADH$_2$ couple in XDH, $-340 \text{ mV}$ (20), is favorable for the reduction of NAD, $-335 \text{ mV}$ at pH 7.5 (21). However, the FAD/FADH$_2$ couple in XO, $-255 \text{ mV}$ (22), is too high for efficient reduction of NAD.

This paper investigates the role of the low redox potential of the FAD/FADH$_2$ couple and the salient NAD-binding site of XDH in conferring reactivity toward NAD as an oxidizing substrate. The midpoint potential of the FAD/FADH$_2$ couple was engineered by replacement of the normal FAD with FAD analogs containing different redox potentials. By reversing the redox potentials of the two enzyme forms, it might be possible to reverse the use of NAD as an oxidizing substrate. The low potential 1-deaza-FAD, $-280 \text{ mV}$ for the unbound flavin, (23) was used to prepare 1-deaza-XO, an oxidase-type enzyme with a low FAD/FADH$_2$ midpoint potential. Previous work of Hille and Massey (24) has shown that 1-deaza-XO has a low flavin potential of $-340 \text{ mV}$ at the higher pH, 8.5. The recently synthesized 8-CN-FAD has a redox potential of $-50 \text{ mV}$ (25). Preparation of 8-CN-XDH resulted in a dehydrogenase-type enzyme with an FAD/FADH$_2$ potential predicted to be too high for efficient NAD reduction. The flavin potentials of these substituted enzymes were determined relative to the 2Fe/2S centers by reductive titrations or by reduction in the presence of suitable redox dyes. Steady-state kinetics of the xanthine/NAD and xanthine/oxygen activities were measured to assess any

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1 The abbreviations used are: XDH, xanthine dehydrogenase; XO, xanthine oxidase; 2Fe/2S, iron-sulfur center; fr. fraction.
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changes in the preferred oxidizing substrate. As NAD binding experiments have focused on spectral changes of the enzyme on addition of NAD, NAD binding was also assessed by measuring the concentration of unbound NAD in mixtures of NAD and oxidized XO or XDH and by isothermal calorimetry. Note that NAD binding to reduced XDH and XO is more catalytically relevant, but it is not possible to measure in the case of XO. Differences in substrate binding and reactivity are discussed.

MATERIALS AND METHODS

Preparation of Artificial FAD-substituted Enzymes—Samples of deflavo XO were prepared by the method of Komai et al. (23). The preparation of 1-deaza-FAD has been described previously by Spencer et al. (23). The synthesis and characterization of 8-CN-FAD was performed by Murthy and Massey and has recently been described (25). Immediately before use, XDH samples were incubated for 1 h at 25 °C with 2.5 mM dithiothreitol. Dithiothreitol was removed by desalting on a Sephadex G-25 column. All experiments were performed at 25 °C in 50 mM potassium phosphate, pH 7.5, 0.3 mM EDTA.

Preparation of Artificial FAD-substituted Enzymes—Samples of deflavo XO were prepared by the method of Komai et al. (23). Deflavo XO was prepared in the same manner but with the addition of 2.5 mM dithiothreitol at all stages. The visible spectra and activity of deflavo enzymes indicated greater than 95% removal of FAD. Reconstitution of holoproteins was performed by incubating deflavo enzyme (approximately 20 μM) with a 3-fold excess of the artificial FAD for at least 30 min on ice. Excess flavin was removed by repeated concentration and dilution in a Centricon-100 spin concentrator. Reconstitution of deflavo XO and XDH with normal FAD ensured that native activity could be recovered.

Anaerobic Titrations—Samples of 1-deaza-FAD-substituted enzyme were purged of oxygen in anaerobic cuvettes, as described previously (9). Photo reductions (26) were performed by careful irradiation of anaerobic enzyme in a sample containing 20 mM potassium oxalate and 5-deazafavin equal to 10% of the enzyme concentration. Dithionite titrations were performed by minute additions of an anaerobic sodium dithionite solution (0.5 mg/ml) from a titrating syringe into an anaerobic solution of enzyme.

Titrations are presented as proportionality plots in which the percent absorbance lost at a principally 2Fe/2S wavelength is plotted as a function of the percent absorbance lost at a mostly FAD wavelength. Redox potentials of the enzyme-bound artificial flavins were determined relative to the 2Fe/2S centers by fitting proportionality plots with simulated curves calculated from the Nernst equation, the known redox potentials of 2Fe/2S I and 2Fe/2S II, and the extinction changes of the iron-sulfur and FAD centers. The fraction of each center reduced was calculated in the same way but with the corresponding extinction changes. Concentrations of 2Fe/2S Ired, 2Fe/2S IIred, FADH°, and FADH 2 were calculated from the change in absorbance at 460 nm after the absorbance change, and the extent of reduction of the flavin was estimated from the absorbance change at 600 nm, where XO shows negligible absorbance changes. The extent of reduction of the enzyme was estimated at 492 nm, an isosbestic point for the reduction of indigotrisulfonate. To calculate the difference between the redox potential of indigotrisulfonate and that of the enzyme-bound 8-CN-FAD, the log(ox/red) of the indicator dye was plotted versus the log(ox/red) of the enzyme-bound 8-CN-FAD according to the method of Minneman (27), a method previously utilized for the measurement of the redox potentials of native XDH (20).

1-ml samples of 8-CN-FAD-substituted XO containing an appropriate indicator dye (indigodisulfonate, indigotrisulfonate, or 1-HO-phenazine) plus 3 units of glucose-6-phosphate dehydrogenase plus 2 μM benzyl viologen were purged of oxygen in an anaerobic cuvette with 2 side arms by gentle agitation under a constant stream of oxygen-free argon for 20 min. The visible spectrum was then recorded. To begin reduction of the XDH by an NADPH generating system, 0.1 μM NADP and 5 mM glucose-6-phosphate were tipped in from the side arms. The solution was thoroughly mixed in the main cuvette, and the reaction was followed by recording visible spectra at 25 °C with time as the NADPH generating system slowly reduced the XDH. For indigotrisulfonate, the extent of reduction of the dye was estimated by the decrease in absorbance at 612 nm, where the enzyme gives rise to less than 5% of the total absorbance change under the conditions used, and the extent of reduction of the flavin was estimated at 463 nm, which is isosbestic for the dye reduction. For indigodisulfonate, the extent of reduction of the dye was estimated by the extent of decrease in absorbance at 600 nm, where the enzyme gives rise to less than 5% of the total absorbance change, and the extent of reduction of the flavin was estimated at 445 nm. No apparent absorbance changes because of the dye were subtracted. For 1-hydroxyphenaxine, the extent of reduction of the dye was estimated by the decrease in absorbance at 372 nm. At this wavelength, absorbance changes associated with formation and reduction of the flavin semiquinone of 8-CN-XDH are negligible. Absorbance changes at this wavelength associated with the 2Fe/2S centers of the enzyme were subtracted. The reduction of FADH 2 was monitored at 620 nm, where 1-hydroxyphenazine does not absorb or is either oxidized or reduced.

Steady-state Kinetics—Steady-state kinetics were measured with a Hi-Tech SF-61 and with a Kinetics Instruments stopped-flow spectrophotometer, wherein excellent control of oxygen concentration can be maintained. The method of initial rates was used. Xanthine/oxygen turnover was measured by the increase in urate concentration at 295 nm (Δε = 9,600 M −1 cm −1). Xanthine/NAD turnover was measured anaerobically as the increase in NADH concentration at 340 nm (Δε = 6,200 M −1 cm −1). Concentrations of xanthine (2–80 μM) and NAD (2–80 μM) or molecular oxygen (31–1220 μM) were varied independently. The enzyme concentration was between 0.1 and 0.5 μM. The enzyme concentration was corrected for inactive enzyme; the fraction of active enzyme was measured as the fraction of absorbance lost within a few s on anaerobic addition of 200 μM xanthine relative to that lost on addition of excess sodium dithionite. Assays with NAD analogs were performed at fixed concentrations of 100 μM xanthine and 500 μM pyridine dinediol. The wavelengths monitored and the extinction changes are as follows: thionicotinamide adenine dinucleotide, Δε 260 = 11,300 M −1 cm −1; acetylpyridine adenine dinucleotide, Δε 260 = 9,100 M −1 cm −1; pyridine aldehyde adenine dinucleotide, Δε 260 = 9,300 M −1 cm −1 (28).

NAD Binding—Native XO or XO (80 μM) was mixed with varying concentrations of NAD at 25 °C. The sample was put in a Centricon-30 spin concentrator, and was centrifuged at 4,000 rpm for 30 min. The concentration of free ligand was taken as the concentration of NAD that passes through the filter. Samples run without enzyme indicated that no NAD was retained by the Centricon filter. NAD was measured by its UV spectrum (ε 260 = 17,800 M −1 cm −1). NAD binding was also measured by isothermal titration calorimetry, as described by Wiseman et al. (29) using enzyme concentrations in the range 100–220 μM. These measurements were kindly made by Dr. Bruce A. Falley, employing a Calorimetry Sciences Corp. (Provo, Utah) 4200 instrument.

\[ E_\text{FADH/FADH}_2 \] This method relies on the assumption that the potentials of the 2Fe/2S centers are unchanged by flavin substitution. The method is accurate insofar as the potential of the FAD is not too far removed from the 2Fe/2S potentials \( E_\text{FADH/FADH}_2 = -310 \text{ mV} \) and \( E_\text{FADH/FADH}_2 = -235 \text{ mV} \).
RESULTS

Preparation and Properties of Artificial FAD-substituted Enzymes—Deflavo XO and XDH were prepared by the method of Komai et al. (6). The visible spectrum as well as activity assays indicated that greater than 95% of the FAD had been removed (not shown). Mixing deflavo XO with 8-CN-FAD and 1-deaza-FAD yielded enzymes with near-complete flavin reconstitution. The addition of 8-CN-FAD and 1-deaza-FAD to deflavo XDH also resulted in near-complete binding, as shown below.

The spectrum of 8-CN-FAD is not significantly changed on binding to deflavo XO or XDH. The extinction at 450 nm of 8-CN-FAD is 9,990 M⁻¹ cm⁻¹ (25). The spectrum of 8-CN-XO minus that of deflavo XO has an extinction difference of 8,800 M⁻¹ cm⁻¹, indicating that only 88% of the 8-CN-XO contains bound flavin. The extinction difference at 450 nm because of 8-CN-FAD binding to deflavo XDH is 9,500 M⁻¹ cm⁻¹, indicating 95% bound flavin in the 8-CN-XDH sample. After reconstitution with 8-CN-FAD, the excess flavin was removed from the enzyme samples in a Centricon-100 concentrator. The spectrum of the initial material (enzyme + excess flavin) minus that of the free 8-CN-FAD that had passed through the filter confirmed the extinctions reported here. Spectra of 8-CN-XO and 8-CN-XDH are shown in Figs. 1, A and B, respectively.

The spectra of 1-deaza-XO and 1-deaza-XDH are identical to that reported previously for 1-deaza-XO (24). The difference spectra of enzyme-bound minus deflavo enzyme indicated the extinction at 550 nm of bound 1-deaza-FAD to be 9,150 M⁻¹ cm⁻¹ for 1-deaza-XO and 9,310 M⁻¹ cm⁻¹ for 1-deaza-XDH. These values are quite close to that of 9,700 M⁻¹ cm⁻¹ reported for 1-deaza-XO (24) and to that of 8,700 M⁻¹ cm⁻¹ for the free 1-deaza-FAD (30). These spectral data indicate near-complete binding of 1-deaza-FAD to deflavo XO and XDH. Again, the spectrum of enzyme with excess 1-deaza-FAD minus that of the unbound 1-deaza-FAD support these extinctions. Spectra of 1-deaza-XO and 1-deaza-XDH are shown in Figs. 5, A and B, respectively.

Reductive Titrations and Determination of Flavin Redox Potentials—The redox potentials of the bound artificial flavins were determined to ensure that the FAD potential had been shifted in the desired direction and amount. Enzyme samples were reduced in very small increments by photo-reduction, by sodium dithionite, or by an NADPH-generating system. Sufficient time (30 to 60 s) was given between irradiations or additions to ensure equilibration. For the 1-deaza-FAD-substituted enzymes, the 2Fe/2S centers were used as redox indicators and are expected to be reduced before the flavin. For the higher
potential 8-CN-FAD-substituted enzymes, dye equilibration studies were carried out, as the 2Fe/2S centers were expected to be of too low a potential to be of use as redox indicators.

Spectra from the reduction of 7.7 μM 8-CN-XO by sodium dithionite are shown in Fig. 1A. In addition to spectra of the oxidized and reduced enzyme, the spectrum containing the maximally obtained FADH\(^{\circ}\) is given. The peak at 414 nm indicates this to be the anionic form of 8-CN-FAD semiquinone (25). Only about 12% of the semiquinone is stabilized, because this species has an extinction increase relative to oxidized 8-CN-FAD of 60 mM (25). This is evident from the spectra in Fig. 1A at that the stage of maximal semiquinone formation there is no reduction of 2Fe/2S centers, because there is no change in the spectrum at wavelengths greater than 520 nm. The FAD/FADH\(^{\circ}\) potential is therefore much higher than that of the 2Fe/2S centers. Identical results are obtained by photo-reduction. Extinction changes for this reduction are given in Table I.

Spectra from the photoreduction of 9.6 μM 8-CN-XO and 20 μM indigotrisulfonate are shown in Fig. 2. The amounts of oxidized and reduced indigotrisulfonate and the amounts of oxidized and reduced enzyme-bound 8-CN-FAD were estimated as indicated under “Materials and Methods.” The log(ox/red) for the indigotrisulfonate was plotted versus the log(ox/red) for the flavin (Fig. 2, inset). An initial slope of 2 corresponds to the small amount of observed semiquinone formation, which is followed by a slope of 0.9 as compared with a theoretical slope of 1 for the 2-electron reduction of the flavin. The plot gives a potential of ~90 mV for the 2-electron reduction of the enzyme-bound 8-CN-FAD. This figure represents a decrease in the potential of 8-CN-FAD of 40 mV on binding to XO, a figure in reasonable agreement with decreases of 47 mV and 50 mV on the binding of native FAD and 1-deaza-FAD, respectively, to XO (Table II).

Spectra from the dithionite reduction of 14 μM 8-CN-XDH are shown in Fig. 1B. The most notable difference with respect to the reduction of 8-CN-XO is that the semiquinone formed is the neutral semiquinone, as seen by the extinction increases from 550 to 650 nm (25). This is entirely consistent with the destabilization of anionic flavins in XDH (17, 18). A plot of \(A_{460}^{\text{versus}} A_{290}\) obtained from the reduction of a 7.1 μM sample of 8-CN-XDH using a NADPH-generating system (inset, Fig. 1B) indicates that, in contrast with the low yield of semiquinone observed during the reduction of 8-CN-XO, 70% of the semiquinone is stabilized. This compares to a stabilization of a large proportion (90%) of the semiquinone obtained during the reduction of XDH-containing native FAD (20). A 70% stabilization of the semiquinone implies that the FAD/FADH\(^{\circ}\) and the FADH/FADH\(_{2}\) potentials are separated by 90 mV.

To determine the potential of the 8-CN-FAD/FADH\(^{\circ}\) couple in XDH, the enzyme was reduced anaerobically using an NADPH-generating system (see “Materials and Methods”) in the presence of 20 μM indigodisulfonate (\(E_m\) at pH 7.5 = −140 mV) and in a separate experiment in the presence of 20 μM indigotrisulfonate (\(E_m\) at pH 7.5 = −140 mV)

### Table I

| Enzyme       | \(\Delta_{\Delta \text{FAD/FADH}}\) (at 2Fe/2S λ) | \(\Delta_{\Delta \text{FAD/FADH}}\) (at 2Fe/2S λ) | \(\Delta_{\Delta \text{FAD/FADH}}\) (at 2Fe/2S λ) | \(\Delta_{\Delta \text{FAD/FADH}}\) (at 2Fe/2S λ) |
|--------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| XO           | 3,400 (550)                                  | 0 (550)                                       | −5,500 (550)                                  | 7,000 (450)                                   |
| 8-CN-XO      | 3,400 (550)                                  | −1,040 (550)                                  | −1,870 (550)                                  | 7,000 (450)                                   |
| 1-deaza-XO   | 7,750 (470)                                  | 2,510 (470)                                   | 1,360 (470)                                   | 3,400 (550)                                   |
| XDH          | 3,400 (550)                                  | 0 (550)                                       | −5,500 (550)                                  | 7,000 (450)                                   |
| 8-CN-XDH     | 3,400 (550)                                  | −1,040 (550)                                  | −2,300 (550)                                  | 7,000 (450)                                   |
| 1-deaza-XDH  | 7,750 (470)                                  | 2,510 (470)                                   | 1,360 (470)                                   | 3,400 (550)                                   |

\(\Delta_{\Delta \text{FAD/FADH}}\) = above values.

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**Fig. 2. Photoreduction of 8-CN-XO in the presence of indigotrisulfonate.** A mixture of 9.6 μM 8-CN-XO and 20 μM indigotrisulfonate was made anaerobic and photoreduced in the presence of 1 μM 5-deazaflavin and 50 mM oxalate as outlined under “Materials and Methods.” Spectra are shown of the following species: solid spectrum, anaerobic 8-CN-XO plus indigotrisulfonate; dotted spectrum, fully reduced 8-CN-XO plus indigotrisulfonate obtained after 13.4 min of irradiation; dashed spectrum, after 3.2 min of irradiation; inset, data obtained at 492 nm for enzyme reduction and at 600 nm for dye reduction as outlined under “Materials and Methods.”
indigotrisulfonate (E_m at pH 7.5 = −96 mV. The amounts of oxidized and reduced dye and the amounts of oxidized and reduced enzyme-bound flavin were estimated as indicated under “Materials and Methods.” The data obtained from the reduction of 8-CN-XDH with indigotrisulfonate were made anaerobic and then was reduced using an NADPH-generating system as outlined under “Materials and Methods.” A mixture of 4.4 μM 8-CN-XDH and 20 μM indigotrisulfonate was made anaerobic and showed a 2-electron acceptor. A slope of 1.2 was obtained. Although the slopes of the plots shown are not easily explained, the measured potentials are all in excellent agreement with results obtained with other flavins (see Table III).

To determine the potential of the 8-CN-FADH⁺/FADH₂ couple in XDH, the enzyme was reduced anaerobically using an NADPH-generating system, as above, in the presence of 25 μM 1-hydroxyphenazine (E_m at pH 7.5 = −187 mV). The amounts of oxidized and reduced dye and the amounts of oxidized and reduced enzyme-bound flavin were estimated as indicated under “Materials and Methods.” A plot of the log(ox/red) of 1-hydroxyphenazine versus the log(ox/red) of the FADH/FADH₂ couple (inset, Fig. 4) gives a value of −198 mV for the FADH⁺/FADH₂ potential. This value is in very close agreement to a theoretical value of −197 mV, assuming an FAD/FADH⁺ potential of −118 mV and 70% stabilization of the semiquinone form. The plot in Fig. 4 is expected to yield a 2-unit slope as both indigotrisulfonate and 1-hydroxyphenazine are 2-electron acceptors. A slope of 1.2 was obtained. Although the slopes of the plots shown are not easily explained, the measured potentials are all in excellent agreement. A lowering of the potential of 8-CN-FAD from −56 to −158 mV on binding to XDH is also in reasonable agreement with results obtained with other flavins (see Table III).

Spectra from a dithionite reduction of 7.4 μM 1-deaza-XO are shown in Fig. 5A. There is only about 10% of the 1-deazaflavin semiquinone species stabilized, which is barely visible as an absorbance increase above 650 nm, where the neutral semiquinone of 1-deaza-FAD has maximal absorbance (31). However, the absorbance increase at 560 nm is very well correlated with the absorbance increase at 580 nm (Fig. 5B). This observation is more clearly in the proportionality plot (Fig. 6) whose upward curvature indicates the flavin to have a lower potential than the 2Fe/2S centers. This is consistent with the results of Hille and Massey at pH 8.5 (24). Fitting these data to the extinctions given in Table I gave optimal correspondence at E°FAD/FADH⁺ = −360 mV and E°FADH⁺/FADH₂ = −290 mV (Fig. 6). The midpoint potential determined here at pH 7.5 of −325 mV is close to that of −340 mV determined at pH 7.5.

| Enzyme       | E_m free FAD mV | E_m enzyme-bound mV | Electron acceptor          | k_cat s⁻¹ | K_m xanthine μM | K_m oxygen μM | K_m NAD μM |
|--------------|----------------|---------------------|----------------------------|-----------|-----------------|---------------|------------|
| XO           | −208           | −255 (22)           | Oxygen (32)                | 13 ± 0.4  | 2 ± 0.5         | 53 ± 2        | 6.9 ± 0.6  |
| 8-CN-XO      | −50 (25)       | −90 ± 4             | Oxygen (7)                 | 0.11 ± 0.01 | 1 ± 0.6   | 250 ± 120     | 1.7 ± 0.3  |
| 1-deaza-XO   | −280 (23)      | −325 ± 10           | Oxygen (9)                 | 0.049 ± 0.012 | 3.7 ± 0.9  | 5.6 ± 1.4     | ND         |
| XDH          | −208           | −340 (20)           | Oxygen (9)                 | 2.1 ± 0.1  | 1.9 ± 0.4        | 65 ± 9        | 7          |
| 8-CN-XDH     | −50 (25)       | −158 ± 11           | Oxygen (18)               | 6.3       | ≤1              | 540 ± 110     | ND         |
| 1-deaza-XDH  | −280 (23)      | −360 ± 14           | Oxygen (18)               | 0.86 ± 0.04 | 1.3 ± 0.4 | 83 ± 4        | 11 ± 1     |

* ND, not determined.

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Fig. 3. Reduction of 8-CN-XDH in the presence of indigodisulfonate using a NADPH-generating system. A mixture of 4.4 μM 8-CN-XDH and 20 μM indigodisulfonate was made anaerobic and then was reduced using an NADPH-generating system as outlined under “Materials and Methods.” Spectra are shown of the following species: solid spectrum, anaerobic 8-CN-XDH plus indigodisulfonate; dotted spectrum, after 27.2 min of reduction; dashed spectrum, after 6 h of reduction. Inset, data obtained at 462 nm for enzyme reduction and at 612 nm for dye reduction as outlined under “Materials and Methods.”

![Fig. 3](image-url)
pH 8.5 (24); the more positive potential of 295 mV would be expected for a pH decrease of 1.0 unit. The potential of 325 mV is similar to that of native XDH, 340 mV (20), indicating that substitution with 1-deaza-FAD does indeed produce a low flavin potential form of XO.

The reduction of 9.8 μM 1-deaza-XDH is shown in Fig. 5B. Except for stabilizing significantly more flavin semiquinone during reduction, 1-deaza-XDH behaves very similarly to 1-deaza-XO. The proportionality plots of the two enzymes are very similar to each other (Fig. 6). The redox potentials determined from fitting these data are $E^\circ_{\text{FAD/FADH}} = 2345$ mV and $E^\circ_{\text{FADH/FADH}_2} = 2375$ mV. The midpoint potential of 260 mV is 20 mV more negative than that of native XDH.

**Steady-state Kinetics of Artificial FAD-substituted XO and XDH**—The ability of the artificial FAD-substituted enzymes to support xanthine/oxygen and xanthine/NAD turnover was measured to assess the role of the flavin midpoint potential in controlling NAD versus oxygen reactivity. Concentrations of both reducing and oxidizing substrates were varied independently. In all cases, Lineweaver-Burk plots displayed sets of parallel lines, consistent with a ping-pong mechanism (not shown). Kinetic constants from steady-state measurements are presented in Table II.

The $k_{cat}$ of 8-CN-XO catalyzed xanthine/oxygen turnover, 12 s$^{-1}$, is quite close to that of native XO, 13 s$^{-1}$ (32). This indicates that the artificial flavin is catalytically active, and it extends the range of data used to study the effect of the flavin

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**Table III**

Activity of NAD analogs with native xanthine oxidase and xanthine dehydrogenase

| Electron acceptor | Redox potential | XO activity | XDH activity |
|-------------------|-----------------|-------------|--------------|
|                   | mV              | %           | %            |
| Oxygen            | +285            | 100 ± 2     | 22 ± 0.4     |
| NAD               | −335            | 2.1 ± 0.2   | 100 ± 6      |
| Thionicotinamide  | −300            | 2.1 ± 0.1   | 120 ± 9      |
| Adenine dinucleotide | −275          | 2.2 ± 0.1   | 102 ± 12     |
| Acetylpyridine    | −245            | 1.8 ± 0.2   | 79 ± 11      |
| Adenine dinucleotide | −245        | 1.8 ± 0.2   | 79 ± 11      |

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**Fig. 5. Reduction of 1-deaza-XO and 1-deaza-XDH.** Panel A, 7.4 μM 1-deaza-XO; panel B, 9.8 μM 1-deaza-XDH. Samples were made anaerobic and reduced by minute additions of 0.5 mg/ml sodium dithionite. Spectra are shown of the following enzyme species: solid spectrum, oxidized enzyme; dotted spectrum, maximal semiquinone; dashed spectrum, reduced enzyme.
midpoint potential on catalysis (24). The high error associated with the oxygen $K_m$ for this turnover as well as the xanthine/oxygen turnover of 8-CN-XDH is because of the $K_m$ value being only slightly lower than the solubility of oxygen at 25 °C, 1.2 mM. 8-CN-XO, like all of the XO forms measured, catalyzes a barely detectable level of xanthine/NAD turnover. This may possibly be because of a small amount, 0.1–2.5%, of contamination of dehydrogenase-type enzyme, because the NAD $K_m$ values of the XO-type enzymes are near those of XDH. The 8-CN-XDH is competent in xanthine/oxygen turnover, $k_{cat}$ of 5.1 s$^{-1}$. However, no amount of xanthine/NAD turnover was detected. The neutral semiquinone species seen on reduction of 8-CN-XDH (Fig. 1B) is evidence that the sample is still a dehydrogenase-type enzyme. Also, 74% of the visible spectrum (relative to dithionite reduction) was bleached on mixing anaerobic 8-CN-XDH with 400 mM aminopyridine adenine dinucleotide. By comparison, the similar NADH reaction with native XO results in no detectable reduction; 2% of absorbance was lost at 15 min. These data demonstrate that 8-CN-XDH is truly a dehydrogenase-type enzyme, despite the lack of xanthine/NAD reductase activity.

For the xanthine/oxygen activity was measured at 260 nm aminopyridine adenine dinucleotide.

NAD activity of the native enzyme at a single set of reaction conditions (7). The low potential of 1-deaza-XDH may result in a greatly decreased fraction of fully reduced flavin during turnover.

**Pyridine Nucleotide Analogs**—To test separately the role of substrate binding and the need for the flavin midpoint potential to be near or below that of its electron acceptor, the ability of native XO and XDH to use a series of pyridine nucleotide analogs was assessed (Table III). Xanthine/oxygen activity was measured at 260 μM oxygen, thus explaining why for XO, xanthine/NAD activity is a higher percent of xanthine/oxygen activity than that in Table II. Redox potentials for pyridine nucleotides were corrected for pH 7.5 by adding −15 mV (−30 mVpH unit) to the values reported at pH 7 (33). Raising the potential of the electron acceptor as high as −245 mV, more positive than the −255-mV flavin potential of XO (22), has no effect on the xanthine/pyridine nucleotide activity of XO. This supports the conclusion from the 1-deaza-
 XO experiments that favorable thermodynamics are not sufficient for dehydrogenase activity. Raising the potential of the pyridine nucleotide acceptor had only modest effects on the xanthine/pyridine nucleotide activity of XDH. This is not surprising, as reduction by xanthine is known to be the slow step in catalysis (34).

NAD Binding to XDH and XO—The flavin spectrum of oxidized XDH is known to be perturbed on the addition of the NAD analog amonopyridine adenine dinucleotide (Refs. 18 and 19 and Fig. 7B), indicating the pyridine nucleotide binds in close proximity to the FAD. Only minute changes ($\Delta \epsilon_{450} = 600 \text{ m}^{-1} \text{ cm}^{-1}$) are observed on the addition of 2.5 mM NAD to 20 $\mu$m XDH, although NAD has a much larger perturbation on the spectrum of chicken liver XDH (19). No spectral changes were observed on the addition of either compound to oxidized XO (Fig. 7A), indicating that NAD does not bind to XO adjacent to the FAD. NAD binding by nonspectral methods was performed to distinguish whether XO has a disrupted NAD-binding site or if there is a binding site and it is not in the vicinity of the FAD. The best comparison would be that of NAD binding to the reduced enzymes. A $K_d$ of NAD binding to fully reduced XDH of 25 $\mu$m has been measured kinetically (35). The addition of NAD to photo-reduced XO results in small absorbance increases ($\Delta \epsilon_{450} = 1,940 \text{ m}^{-1} \text{ cm}^{-1}$, $\Delta \epsilon_{450} = 2,040 \text{ m}^{-1} \text{ cm}^{-1}$) throughout the visible spectrum, consistent with oxidation of a small fraction of XO and not consistent with any FADH$_2$:NAD complex (not shown). As mixing reduced XO and NAD provides no information about binding, binding of NAD to the oxidized enzymes was measured. In initial experiments, 80 $\mu$m solutions of oxidized XDH and XO were each mixed with varying concentrations of NAD from equivomolar to 1 $\mu$m. Measurements of the unbound NAD concentration was obtained from the spectrum of the flow-through when the sample was centrifuged in a Centricon-30 concentrator. Using this method, a $K_d$ of 280 ± 145 $\mu$m was obtained for the binding of NAD to XO, but no evidence could be obtained indicating binding of NAD to XO. NAD binding to XDH was also measured by a completely different analytical method, that of isothermal titration calorimetry (29), yielding a $K_d$ of 160 ± 40 $\mu$m (results not shown). Again, in a separate experiment, no indication of NAD binding to the oxidase form could be obtained.

**DISCUSSION**

The goal of the present work was to assess the role of the low flavin midpoint potential and of NAD binding in determining specificity toward oxidizing substrates for XO and XDH. The flavin midpoint potential was engineered by preparing enzyme samples substituted with artificial flavins. FAD redox potentials of these enzyme forms indicated that the desired changes in potential had indeed occurred. Preparation of 8-CN-XDH resulted in an enzyme with a flavin midpoint potential of −158 mV, predicted to be too high to efficiently reduce NAD, −335 mV at pH 7.5 (21). This enzyme was completely lacking in xanthine/NAD activity even though several experiments indicated it was still dehydrogenase-type. A low flavin potential is certainly necessary for NAD reactivity. Preparation of 1-deaza-XO yielded an oxidase-type enzyme with a flavin midpoint potential close to that of native XDH. Although as functional as an oxidase, 1-deaza-XO has no more dehydrogenase activity than does native XO. This residual activity could be because of a small amount, 0.1–2.5%, of contaminating dehydrogenase-type enzyme. Measuring the activity of native XO and XDH in assays using NAD analogs of higher redox potentials also demonstrated that only dehydrogenase-type enzyme could utilize pyridine nucleotides as an oxidizing substrate. These data indicate that favorable thermodynamics are necessary, but not sufficient, for dehydrogenase activity. Clearly NAD binding is an essential factor.

The visible spectrum of XO is not perturbed upon the addition of NAD or of aminopyridine adenine dinucleotide, unlike that of XDH (19, 18). Also, NAD does not inhibit xanthine/oxygen turnover of XO. At 100 $\mu$m xanthine, 260 $\mu$m oxygen, and 500 $\mu$m NAD, xanthine/oxygen turnover proceeds at 99.6% the rate obtained in the absence of NAD (not shown). Under these conditions, XDH-catalyzed xanthine/oxygen turnover would be completely inhibited in favor of xanthine/NAD turnover (9). The existence of an NAD binding site in XDH, but not in XO, was found by measuring the concentration of unbound NAD in mixtures of NAD and XO or XDH and independently by isothermal titration calorimetry. Values of $K_d$ for NAD binding to oxidized XDH 280 ± 145 $\mu$m and 160 ± 40 $\mu$m, respectively, were measured using these methods. Thus, binding of NAD to XO is relatively weak, with a $K_d$ ~ 220 $\mu$m. Nishino and Nishino (19) measured a $K_d$ of 310 $\mu$m for NAD binding to oxidized chicken liver XDH (19). NAD binding to the two-electron reduced state of XDH has previously been modeled to be approximately 200 $\mu$m, supporting the concept of weak NAD binding to more oxidized enzyme forms (35). These binding data, along with the lack of spectral perturbation on pyridine nucleotide addition, the lack of xanthine/NAD activity of 1-deaza-XO, and the insensitivity of xanthine/oxygen activity to the presence of NAD indicate that the conformational changes that convert XDH to XO also disrupt the NAD-binding site in XO. The binding experiments reported here are with the oxidized enzymes. NAD binding to reduced XO and XDH is more catalytically relevant although not possible to measure for XO. Note that oxygen binding is not an issue in determining substrate specificity: XDH has been shown to react with oxygen by second-order reactions (9), and there is no good evidence for oxygen binding to XO or any other flavoprotein oxidase (36).

NAD binding to oxidized XDH, $K_d$ ~ 220 $\mu$m, is approximately 10-fold weaker than to fully reduced enzyme, $K_d$ of 25 $\mu$m (34). Using these $K_d$ values and −340 mV as the flavin midpoint potential of unbound XDH (20), a flavin midpoint potential of −370 mV can be calculated for NAD-bound XDH based on a thermodynamic box (Scheme 1). This 30-mV lowering in flavin potential may facilitate reduction of NAD. This might be at the expense of decreasing electron density at the flavin and increasing it at the iron and molybdenum centers. But, in oxidation with NAD (35) and in reduction with NADH (34), intramolecular electron transfer into and out of the flavin appear to only happen in the absence of bound NAD. Intramolecular electron transfer to make FADH$_2$ is only slightly disfavored, $K_m = 0.12$, before NAD binding. Results from the oxidative half-reaction with NAD (35) indicate that formation of FADH$_2$ precedes NAD binding. Thus the energy of substrate binding is used to lower the midpoint potential of the FADH$_2$, shifting the difference between FAD and NAD potentials from 5 to 35 mV. The $K_d$ of 310 $\mu$m for NAD binding to oxidized chicken liver XDH (19) is also much higher than that measured kinetically for the fully reduced enzyme, 80 $\mu$m (37).

Using samples of XO substituted with artificial flavins having a wide range of midpoint potentials, Hille and Massey (24) found a sigmoidal relationship between increases in $V_{max}$ and the increasing fraction of reduced flavin in the steady state. In the present study, samples of native XDH, 1-deaza-XDH, and 1-deaza-XO all fall in the minimal $V_{max}$ region of this curve with respect to their xanthine/oxygen turnover. This curve predicts both 8-CN-XO and 8-CN-XDH should have $k_{cat}$ values of 16 s$^{-1}$. At 12 s$^{-1}$, 8-CN-XO is close to this curve, whereas at 5.1 s$^{-1}$, 8-CN-XDH clearly behaves differently. Note that the study of Hille and Massey was performed at a higher pH, 8.5. Despite having a flavin potential poised for NAD reduction
(20), a highly specific NAD-binding site (18, 19, 34, 37) and strict preference for NAD over oxygen as a substrate (9), native XDH still behaves like a low potential oxidase-type enzyme with respect to its xanthine/oxygen turnover (in the absence of NAD). Dehydrogenase activity requires a number of specific structural and chemical properties; oxidase activity merely requires the presence of a reduced flavin. This teleological argument suggests that the XO form may have little physiological function.

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