Kinetic Isotope Effects and Electron Transfer in the Reduction of Xanthine Oxidoreductase with 4-Hydroxypyrimidine

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Isolated from bovine milk, xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are two interconvertible forms of the same protein, differing in the number of protein cysteines versus cystines. Most differences between XO and XDH are localized to the FAD center, the site at which the oxidizing substrates NAD and molecular oxygen react. A comparative study of the reduction of XO and XDH has been performed to assess differences in reactivity of the molybdopterin site, as well as subsequent electron-transfer events from molybdenum to 2Fe/2S and FAD centers. The compound 4-hydroxypyrimidine (4-OH-P) was chosen as reducing substrate because its higher $K_m$ value raised the possibility of binding weak enough to measure kinetically, and its high $k_{cat}$ value could allow detection of intramolecular electron-transfer reactions.

As measured by stopped flow spectrophotometry, XO and XDH react with the first equivalent of 4-OH-P via similar mechanisms, differing in the magnitude of rate and dissociation constants. Using [2-2H]4-OH-P as substrate, a $k/K_p$ isotope effect of 1.9 to 2.3 suggests that movement of the hydrogen abstracted from substrate appreciably limits the rate of initial enzyme reduction from Mo(VI) to Mo(IV). Monitoring the visible spectrum of the enzymes, the first observed step is reduction of a single 2Fe/2S center and presumably re-oxidation of Mo(IV) to Mo(V). This suggests a common pathway for electron transfer involving reduction of a 2Fe/2S center prior to reduction of the second 2Fe/2S and FAD centers. Rates of the first electron transfer from molybdenum to the 2Fe/2S center are rapid, 290 s$^{-1}$ with XO and 180 s$^{-1}$ with XDH, and are consistent with rates measured by flash photolysis (Walker, M. C., Hazzard, J. T., Tollin, G., and Edmondson, D. E. (1991) Biochemistry 30, 5912–5917) allowing discrete observation of the electron-transfer reactions that occur during turnover. This step also exhibits a modest primary kinetic isotope effect of 1.5 to 1.6 when [2-2H]4-OH-P is used, possibly due to deprotonation of the molybdenum center prior to electron transfer. A second one-electron transfer, presumably oxidizing Mo(V) to Mo(VI), follows in a step coincident with product dissociation, consistent with a role for product release in controlling electron transfer events. The kinetics of this complex system are described and interpreted quantitatively in models that are consistent with all the data.

Xanthine oxidase (XO)$^1$ from bovine milk exists as a dimer containing 1 molybdopterin, 1 FAD, and 2 spinach ferredoxin-type 2Fe/2S centers per 150-kDa subunit (1, 2). XO is involved in purine catabolism, oxidizing hypoxanthine to xanthine and xanthine to urate, with concomitant reduction of oxygen. XO functions according to a ping-pong mechanism, with xanthine oxidation occurring at the molybdenum center and O$_2$ reduction occurring at the FAD center (3). The 2Fe/2S clusters appear to function as electron sinks, mediating electron transfer from molybdenum to FAD. According to the rapid equilibrium hypothesis of Olson and coworkers (4), the rate-limiting step in catalysis is the release of the product urate, following which electrons rapidly re-equilibrate from molybdenum to the other enzyme cofactors according to the redox potentials of each center. This model proposes that intramolecular electron transfer is much faster than turnover ($k_{cat}$ of 35 s$^{-1}$ at 25 °C, pH 8.5 (4)), a notion supported by pH jump (5), flash photolysis (6), and pulse radiolysis (7) experiments in which rates of electron redistribution of 100–330 s$^{-1}$ were measured under the same conditions.

Pretreatment of XO with thiold reducing agents such as di-thiothreitol or glutathione results in conversion of XO to xanthine dehydrogenase (XDH), which prefers NAD as oxidizing substrate (8–10). The reaction is reversible; addition of oxidizing agents such as pyridine disulfide or glutathione disulfide converts XDH to XO (9, 10). Unlike chicken liver XDH which cannot be reversibly converted to an oxidase, the enzyme from bovine milk represents a unique system for studying how protein conformation modulates function. Bovine milk XDH has many properties in common with XDH from chicken liver including an NAD binding site, stabilization of the blue, neutral FADH$^+$ during reduction, and destabilization of anionic flavins (10–14). In contrast, the XO form of the milk enzyme shows no perturbation of the FAD spectrum by pyridine nucleotides, little FADH$^+$ is observed during reduction, and anionic flavins are stable at neutral pH (1, 10). Although the redox potential of the FAD/FADH$^+$ couple is unchanged between milk XO and XDH, the FADH$^+$/FADH$_2$ couple is 180 mV lower in XDH than XO (15, 16). This change in the FAD redox potential results in much greater stabilization of the FADH$^+$ species, and it also supplies a thermodynamic explanation for the difference in oxidizing substrates between XO and XDH; the FAD/FADH$_2$ couple in XO has a midpoint potential of −255 mV (16), too high to efficiently reduce NAD at −335 mV (−320 mV at pH 7.0 (Ref. 17) −30 mV per pH unit), whereas the midpoint potential of FAD in XDH is −340 mV (15), low enough to reduce NAD.

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$^1$ The abbreviations used are: XO, xanthine oxidase; XO, $\text{XO}_{\text{nuc}}$, xanthine oxidase reduced by n electrons; $\text{XO}_{\text{nuc}}$, fully-reduced xanthine oxidase; XDH, xanthine dehydrogenase; XDH, xanthine dehydrogenase reduced by n electrons; XDH, fully-reduced xanthine dehydrogenase; 4-OH-P, 4-hydroxypyrimidine; 2Fe/2S, iron-sulfur center; FAD, flavin adenine dinucleotide.
The reaction of the molybdenum center with reducing substrates such as xanthine involves transfer of the C8 proton (probably to the Mo=S sulfido group to give Mo-SH), 2-electron reduction of the metal (from Mo(VI) to Mo(IV)), and formation of a Mo-R (18) or Mo-O-R (19) bond in which R is the C-8 carbon of xanthine, and the oxygen is derived from an oxo, a hydroxyl, or a water ligand. Regeneration of the initial Mo(VI) form requires two-electron oxidation of molybdenum, deprotonation from Mo-SH, and urate dissociation. Oxidation from Mo(IV) to Mo(VI) occurs in two discrete one-electron transfer reactions (20) to the 2Fe/2S and FAD centers. Both mechanisms (18, 19) involve deprotonation from Mo-SH, of the proton derived from xanthine, and ammonia, and ammonia, and xanthine. Because electron re-distribution has been measured to be quite fast (5–7), product release is thought to be slow with electron transfer rapidly following on oxidation of Mo(V) to Mo(VI). In kinetic studies product dissociation and electron re-distribution are observed coincidently (21).

With four redox-active centers in the same protein, XO and XDH are excellent model systems for studying intramolecular electron transfer reactions. Dissection of the events in the reductive half-reaction of XO and XDH with xanthine is complicated by the tight binding of xanthine as well as the similarity of rates observed in different reaction phases (22). The current study focuses on the differences in the reductive half-reactions of XDH and XO with the alternate substrate 4-hydroxyxanthine (4-OH-P). XO oxidizes 4-OH-P to 2,4-dihydroxyxanthine (uracil), in a non-physiological reaction. XO oxidizes 4-OH-P to 2,4-dihydroxyxanthine (4-OH-P). XO oxidizes 4-OH-P to 2,4-dihydroxyxanthine (4-OH-P). XO oxidizes 4-OH-P to 2,4-dihydroxyxanthine (4-OH-P). XO oxidizes 4-OH-P to 2,4-dihydroxyxanthine (4-OH-P).

Xanthine dehydrogenase was purified by the method of Hunt and Massey (10). Xanthine oxidase was purified by the same method with the exclusion of dithiothreitol. The percent functional enzyme was measured prior to use by determining the fraction of absorbance lost at 450 nm within seconds on anaerobic reduction by 200 µM xanthine relative to the total absorbance change after further reduction with an excess of sodium dithionite. Xanthine dehydrogenase samples were incubated with 2.5 mM dithiothreitol for 1 h at 25 °C prior to use.

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Steady-state Kinetics—Steady-state kinetics of xanthine dehydrogenase were performed by the method of initial rates, measuring the change in absorbance at 340 nm as NAD is converted to NADH (ΔE = 6,200 mV cm⁻¹). The stopped flow instrument was used to maintain an anaerobic environment. Xanthine oxidase turnover was also measured by the method of initial rates. The concentration of oxygen was varied by bubbling cuvettes containing buffer and substrate at least 15 min with different mixtures of oxygen and nitrogen. A small volume of enzyme was added to initiate the reaction, following which the cuvette was immediately capped. The reaction was monitored at 260 nm (ΔE = 5,000 mV cm⁻¹) in a Cary 219 spectrophotometer.

Analysis of Rapid-reaction Data—Pre-steady-state data can generally be described according to the following two-step mechanism (Reaction 1) where A is the enzyme, B a substrate, C a Michaelis complex, and D an enzyme-product complex.

\[
A + B \rightarrow C \rightarrow D
\]

Based on the condition that substrate binding is a rapid equilibrium (see Equation 2), i.e. \( k_2 \gg k_3 \).

When the value of \( k_4 \) is significant with respect to \( k_3 \), a plot of \( k_{obs} \) versus substrate concentration will saturate toward \( k_4 \) at high substrate concentration and will approach the y axis at a value equal to \( k_4 \) at low substrate concentration. When \( k_4 \) is insignificant with respect to \( k_3 \), a plot of \( k_{obs} \) versus substrate concentration will exhibit true hyperbolic behavior. The concentration of B that corresponds to the half-maximal rate is equal to the true dissociation constant for B, \( k_4 \), so long as a rapid binding equilibrium holds, i.e. \( k_2 \gg k_3 \). The general case is better described as an apparent \( K_E \) (see Equation 3).

\[
K_E = \frac{k_2 + k_3[B]}{k_4}
\]

In the current study, the signal changes are observed at least one step later than \( k_3 \), above and are better described by a three-step mechanism as shown in Reaction 2.
A solution for the formation of $E$ was derived in the manner of Strickland et al. (27), applying the steady-state condition to $C$ and $D$, and under the condition that $k_3$ and $k_5$ approach zero (Equation 4).

$$h_{ss} = \frac{k_1 k_3 k_3}{k_3 + k_5}$$  \hspace{1cm} (Eq. 4)

Equation 4 still describes a rectangular hyperbola, but interpretation of the maximal rate and dissociation constant must be expressed as $k_{app}$ and $K_{d,app}$ (see Equations 5 and 6) where

$$k_{app} = \frac{k_3 k_3}{k_3 + k_5}$$  \hspace{1cm} (Eq. 5)

$$K_{d,app} = \frac{k_3 (k_3 + k_5)}{k_3 (k_3 + k_5)}$$  \hspace{1cm} (Eq. 6)

As $k_5$ becomes large with respect to $k_3$, $k_{app}$ approaches $k_5$. Equation 6 contains more terms than that of $K_{d,app}$ for the two-step mechanism, Equation 3. Assumption of a rapid-binding equilibrium in the three-step mechanism would still leave terms for $k_3$ and $k_5$ in the expression of $K_{d,app}$. An isotope effect on $k_{app}$ can be interpreted as an effect on $k_3$, $k_5$, or both. Changes in $K_{d,app}$ are best described as a $V(k/K_a)$ effect, as the resulting expression has no contribution from $k_5$.

$$\frac{k_{app}}{K_{d,app}} = \frac{k_3 k_3}{k_3 + k_5}$$  \hspace{1cm} (Eq. 7)

Equation 7 is identical to that for the expression for $V(K_a)$ used to describe isotope effects on steady-state kinetics (28) for a mechanism in which $k_5$ regenerates $A$. The comparison between $V(k/K_a)$ and $V(K_a)$ isotope effects can be extended; both are exhibited due to an effect on $k_5$ that is suppressed by a subsequent slower, irreversible step, $k_6$. The utility of the $V(k/K_a)$ effect is in distinguishing an isotope effect on $k_3$, as Equation 7 does not contain $k_3$. In the case where $k_3$ or $k_5$ is significant, this analysis does not hold, as the $k/K_a$ expression will contain a $k_5$ term.

### RESULTS

**Steady-state Kinetics—Turnover of XDH with 4-OH-P as reducing substrate and NAD as oxidizing substrate was measured by the method of initial rates at 25 °C, pH 7.5. A Lineeweaver-Burk plot of these data (Fig. 1A) indicates parallel lines at low substrate concentration, consistent with a ping-pong mechanism, and converging lines at high substrate concentration, suggestive of a ternary complex mechanism.** A replot of the primary intercepts from Fig. 1A versus reciprocal NAD concentration yielded a straight-line relationship (Fig. 1B), consistent with either steady-state mechanism. Kinetic constants are given in Table I. A replot of the primary slopes from Fig. 1A versus reciprocal NAD concentration (Fig. 1B) resulted in a curve that could be fit well with a hyperbola that corresponds to a $K_m$ for 4-OH-P of 32 ± 7 μM at low NAD and a $K_m$ that approaches zero at high NAD. This phenomenon can be explained by a change in mechanism from ping-pong to ternary complex, depending on the reaction conditions. The reason for this novel ternary complex behavior is evident from the pre-steady-state experiments discussed below. Since oxidation of 4-OH-P to uracil must involve removal of the 2-hydrogen atom, [2-3H]4-OH-P was synthesized, and the steady-state kinetic isotope effects were measured. Lineeweaver-Burk plots of [2-3H]4-OH-P/NAD turnover also exhibit convergent lines at high substrate concentration (not shown). It is clear from the data in Table I that there is no isotope effect on $k_{cat}$ and $K_m$. However, the observed $K_m$ of 55 ± 7 μM for [2-3H]4-OH-P gives a $V(K_m)$ isotope effect of 1.7 ± 0.4. To facilitate interpretation, the spectrum of XDH during catalysis was measured by anaerobically mixing 40 μM oxidized XDH with an equal volume of 1.5 mM 4-OH-P and 1 mM NAD in a diode array stopped flow instrument, at 25 °C and pH 7.5. The spectrum of XDH during the turnover portion of the reaction is most consistent with a two- to three-electron-reduced enzyme species (not shown).

Lineweaver-Burk plots of XO-catalyzed oxidation of 4-OH-P with O2 as oxidizing substrate did give parallel lines (not shown). Kinetic constants are given in Table I. Like XDH, XO shows no significant isotope effect on $k_{cat}$ and $K_m$ for the oxidizing substrate, but XO does show a significant isotope effect

| Table I | Steady-state Parameters for XDH and XO Catalysis with [2-3H]4-OH-P and [2-3H]4-OH-P |
|---------------------------------|---------------------------------|
| Enzyme  | Substrate | $k_{cat}$ | $V/K_m$ | $K_m$ 4-OH-P | $V(K_m)$ 4-OH-P | $k_{cat}$ oxidizing substrate | $V(K_m)$ oxidizing substrate |
|--------|-----------|----------|--------|-----------|--------------|------------------|------------------|
| XDH    | 4-OH-P    | 24 ± 1   | 1.0 ± 0.1 | 32 ± 7 | 1.7 ± 0.4 | 20 ± 3 | 1.1 ± 0.2 |
|        | [2-3H]4-OH-P | 24 ± 1   | 1.0 ± 0.1 | 55 ± 7 | 2.5 ± 0.4 | 120 ± 6 | 0.96 ± 0.14 |
| XO     | 4-OH-P    | 32 ± 1   | 1.0 ± 0.1 | 27 ± 4 | 2.5 ± 0.4 | 65 ± 1 | 110 ± 20 |
|        | [2-3H]4-OH-P | 31 ± 1   | 1.0 ± 0.1 | 65 ± 1 | 2.5 ± 0.4 | 65 ± 1 | 110 ± 20 |

Reactions were performed at 25 °C, pH 7.5. Values were determined by the method of initial rates, independently varying the concentrations of both oxidizing and reducing substrates. For XDH, NAD is the oxidizing substrate and for XO, O2 is the oxidizing substrate.
on \( K_a \) for 4-OH-P \( (\text{mM}/K_a = 2.5 \pm 0.4) \). The spectrum of XO during catalysis was estimated in enzyme-monitored turn-over experiments by reacting in the stopped flow instrument 1.6 \( \mu \text{M} \) XO with 50 \( \mu \text{M} \) 4-OH-P over a range of oxygen concentrations from 130 to 740 \( \mu \text{M} \). The extinction at 450 nm measured in the steady-state portion (0.1 to 2 s) ranged from 16,800 \( \text{M}^{-1} \text{cm}^{-1} \) at 130 \( \mu \text{M} \) oxygen to 23,800 \( \text{M}^{-1} \text{cm}^{-1} \) at 740 \( \mu \text{M} \) oxygen, corresponding approximately to a range from \( \text{XO}_{5e} \) to \( \text{XO}_{6e} \), respectively (4). The amount of reduction observed at the onset of the steady-state phase is a function of the initial ratio of reducing and oxidizing substrates. This suggests that the relative rates of reduction and oxidation for XO are close in magnitude.

**Spectral Intermediates in the Reductive Half-reaction with Excess Substrate**—The reductive half-reactions of XDH and XO were studied in detail with 4-OH-P to better evaluate the nature of each step observed in reduction. Reactions were performed by mixing anaerobic, oxidized enzyme in a stopped flow instrument with anaerobic solutions containing the reducing substrate. In the absence of oxygen or NAD, \( E_{\text{ox}} \) is converted to \( E_{\text{red}} \) in a reaction involving only the first half of the normal catalytic cycle. Since XDH and XO can accept up to six electrons from reducing substrates, three distinct two-electron reduction steps utilizing a total of 3 eq of substrate are required to convert \( E_{\text{ox}} \) to \( E_{\text{red}} \). As the molybdenum undergoes no significant absorbance changes on reduction, these spectrophotometric experiments observe reduction of only the 2Fe/2S and FAD centers. Also, as molybdenum is the initial site of reduction by 4-OH-P, observed absorbance changes occur subsequently to the first two-electron reduction.

To determine the enzyme species that are involved in reduction, 20 \( \mu \text{M} \) XDH was reacted anaerobically with 500 \( \mu \text{M} \) 4-OH-P (final concentrations) in the diode array stopped flow instrument, and spectra were recorded from 1.2 ms to 12 s. Singular value decomposition of the spectra with the Specfit program indicated that two intermediates were present in the reaction. Global analysis of the data by a four-species consecutive model yielded spectra of the oxidized and reduced enzyme species and of the two intermediates (Fig. 2A). Observed rates for the three reaction phases in their order of appearance are 99, 26, and 7.8 \( \text{s}^{-1} \). The spectrum of the first intermediate (spectrum 2, Fig. 2A) corresponds to reduction of 1.5–1.8 2Fe/2S centers as apparent from the loss of absorbance throughout the visible region (3). This spectrum most closely resembles an XDH_{3e} species. However, XDH_{3e} is predicted to have 0.68 electrons on FAD as FADH^{2+} and only 1.21 electrons on 2Fe/2S, based on equilibrium measurements (15). Observation of a non-equilibrium distribution of electrons in intermediate 1 implies that intramolecular electron transfer has either not reached equilibrium or is influenced by an enzyme-bound ligand. The spectrum of the second intermediate (spectrum 3, Fig. 2A) shows further reduction of the 2Fe/2S centers as well as formation of a blue, neutral FADH^{2+} species characterized by the increases in long wavelength absorbance (29). The extinction increase at 620 nm of 3,800 \( \text{M}^{-1} \text{cm}^{-1} \) corresponds to near-complete formation of FADH^{2+}: This intermediate has been assigned as XDH_{4e}, and the observed spectral changes correspond precisely to those expected under equilibrium conditions (15). In the last phase of the reaction, the FADH^{2+} species is converted to FADH_{2} to form XDH_{6e} (spectrum 4, Fig. 2A). This is concluded from the bleaching of the long wavelength absorbance.

Spectral intermediates in the reduction of XO were also measured by anaerobically reacting 9.1 \( \mu \text{M} \) XO with 40 \( \mu \text{M} \) 4-OH-P (final concentrations) in the diode array stopped flow instrument. Singular value decomposition indicated that again two intermediates were present in the reaction. Attempts at global fitting of the data to a four-species model with all rates variable were unsuccessful. Analysis with rates fixed to values from single wavelength experiments (below) of \( k_1_{\text{obs}} = 110 \text{ s}^{-1}, k_2_{\text{obs}} = 66 \text{ s}^{-1}, \) and \( k_3_{\text{obs}} = 27 \text{ s}^{-1} \) resulted in the spectra in Fig. 2B. The first intermediate (spectrum 2, Fig. 2B) corresponds closest to \( \text{XO}_{5e} \) with 0.98 2Fe/2S center reduced (and presumably a Mo(V) species) due to the loss of approximately one-half the absorbance above 500 nm. The second intermediate (spectrum 3, Fig. 2B) has absorbance decreases in the 450-nm region and absorbance increases from 490 to 650 nm. This species corresponds to 0.62 FADH^{2+}, as well as 1.04 2Fe/2S center reduced, indicating a second \( \text{XO}_{5e} \) species formed by re-equilibration of electrons from intermediate 1. The last reaction phase involves a loss of FADH^{2+} and 2Fe/2S absorbance as intermediate 2 is converted to \( \text{XO}_{6e} \) (spectrum 4, Fig. 2B). Note that this step involves \( \text{XO}_{6e} \) being converted directly to \( \text{XO}_{6e} \) without an observed \( \text{XO}_{5e} \) intermediate.

**Kinetics of XDH Reductive Half-reaction**—More reliable values for rate constants in the reductive half-reaction were obtained by anaerobically reacting XDH with varying concentrations of 4-OH-P and collecting data at individual wavelengths. Reaction traces and simulations of the data at 450, 550, and 620 nm are shown (Fig. 3A). XDH data were fit to a Marquardt-Levenberg algorithm with Program A and required three exponentials to adequately fit all data. Observed rates for the traces shown at a final concentration of 500 \( \mu \text{M} \) 4-OH-P are 96, 26, and 9.1 \( \text{s}^{-1} \) for the first, second, and third phases, respectively. These values are in good agreement with those deter-
FIG. 3. Reductive half-reaction of XDH and XO with excess 4-OH-P, plot of absorbance versus time and simulations of the data. Reactions were carried out at 25 °C, pH 7.5. XDH, 9.8 μm active, was mixed anaerobically with an equal volume of 1 mm 4-OH-P, and 9.7 μM XO was mixed with 1 mm 4-OH-P. Traces were monitored at individual wavelengths in a stopped flow instrument. Absorbance traces (curves) are shown along with simulations to the data (solid symbols): circles, at 450 nm; squares, 550 nm; and triangles, 620 nm. Data were fit to a three-exponential consecutive mechanism with Program A (not shown). A, reduction of XDH. Values of k_{obs} for XDH are 96, 26, and 9.1 s\(^{-1}\) for phases 1, 2, and 3, respectively. Simulations of the XDH data were calculated with Program A according to the model given in Scheme 1. Steps described by k_{2}, k_{3}, k_{4}, and k_{5} were not included in the simulation model. A rapid equilibrium was assumed for all substrate binding steps. All other steps appear to be irreversible under the reaction conditions. Setting any reverse rate constant close in magnitude to its correspondingly forward rate constant significantly altered the k_{obs} values determined by fitting the simulation. Rate constants used were k_{1} = 2.5 × 10^{7} M^{-1} s^{-1}, k_{2} = 210.0 s^{-1}, k_{3} = 180 s^{-1}, k_{4} = 26 s^{-1}, k_{5} = 2.5 × 10^{8} M^{-1} s^{-1}, k_{6} = 2100 s^{-1}, k_{7} = 180 s^{-1}, k_{8} = 26 s^{-1}, k_{9} = 3.7 × 10^{8} M^{-1} s^{-1}, k_{10} = 1900 s^{-1}, k_{11} = 14 s^{-1}. Simulations were corrected for the absorbance of inactive enzyme. All extinction coefficients are reported as M\(^{-1}\) cm\(^{-1}\). Extinction coefficients used at 450 nm were 37,800 for species A–C, 29,700 for D and E, 29,200 for F–H, 18,600 for I–K, and 15,800 for L and M. Extinction coefficients used at 550 nm were 12,500 for species A–C, 8,800 for D and E, 6,270 for F–H, 9,700 for I–K, and 6,270 for L and M. Extinction coefficients used at 620 nm were 2,270 for species A–C, 1,920 for D and E, 1,620 for F–H, 3,970 for I–K, and 1,420 for L and M. B, reduction of XO. Values of k_{obs} for XO are 250, 250, and 69 s\(^{-1}\), in order of appearance. Simulations of the XO data were calculated with Program A according to the model given in Scheme 2. The step described by k_{2} was not included in the simulation model. A rapid equilibrium was assumed for all substrate binding steps. All other steps appear to be irreversible as determined from simulations. Rate constants used were k_{1} = 2.5 × 10^{7} M^{-1} s^{-1}, k_{2} = 410 s^{-1}, k_{3} = 290 s^{-1}, k_{4} = 280 s^{-1}, k_{5} = 2.5 × 10^{8} M^{-1} s^{-1}, k_{6} = 410 s^{-1}, k_{7} = 3.7 × 10^{8} M^{-1} s^{-1}, k_{8} = 1900 s^{-1}, k_{9} = 14 s^{-1}. All extinction coefficients are reported as M\(^{-1}\) cm\(^{-1}\). Simulations were corrected for the absorbance of inactive enzyme. Extinction coefficients used at 450 nm were 37,800 for species A–C, 33,000 for D, 29,000 for E and F, and 17,500 for G. Extinction coefficients used at 550 nm were 12,500 for species A–C, 11,500 for D, 12,300 for E and F, and 8,800 for G. Extinction coefficients used at 620 nm were 3,200 for species A–C, 2,410 for D, 3,700 for E and F, and 1,640 for G.

The variation of k_{obs} as a function of 4-OH-P concentration is given for XDH in Fig. 4A. The first and third phases of XDH reduction follow saturation kinetics. Equation 4 is valid under the condition that the reaction is essentially irreversible (k_{3} ≫ k_{5} ≫ k_{4}). The close correspondence between the data and the hyperbolic fit in Fig. 4A supports this assumption as data would extrapolate to a positive y intercept value at low substrate concentration if the reaction were reversible (see “Materials and Methods”). Hyperbolic fits yielded the kinetic parameters listed in Table II. The terms k_{app} and K_{d, app} are used because the absorbance changes of chromophores monitored in each reaction phase (steps k_{1}, k_{5}, k_{7}, and k_{11} in Scheme 1) occur subsequent to enzyme reduction at the colorless molybdenum center (steps k_{2} and k_{4} in Scheme 1), which does not undergo significant spectral changes in this reaction. The values for k_{app} and K_{d, app} approach the true microscopic rate and dissociation constants as the steps between substrate binding and each spectral change become very fast. The values for k_{app} for phases 1 and 3 have modest deuterium isotope effects of 1.7 ± 0.2 and 1.6 ± 0.2, respectively, when [2-D]4-OH-P is used as substrate (Fig. 5A). Phase 2 exhibits no isotope effect, k_{4} = 0.98 ± 0.2. Limiting rates of 71 ± 8.5 s\(^{-1}\) for phase 1 and 9.1 ± 0.3 s\(^{-1}\) for phase 3 were found with [2-D]4-OH-P as reductant. The reaction monitored in the first and last phases is transfer of an electron from molybdenum to some other cofactor. Presumably this step is isotopically sensitive due to depletion of the hydrogen derived from substrate prior to electron transfer, in the oxidation of Mo(IV) to Mo(V). When

FIG. 4. Plot of k_{obs} as a function of substrate concentration for the reduction of XDH and XO with excess 4-OH-P. XDH, 4.9 μM active enzyme after mixing, or XO, 4.7 μM active enzyme after mixing, was reacted with varying concentrations of 4-OH-P. All data were fit to a three-exponential consecutive mechanism. Data, solid symbols, shown along with simulated rates, open symbols, determined by fitting simulations of reaction traces as discussed in Fig. 3. A, reduction of XDH. Values from a free fit for phase 2, squares (left-hand scale), showed little concentration dependence and were averaged for a value of 26 ± 4 s\(^{-1}\). Values for phase 1, circles (left-hand scale), and phase 3, triangles (right-hand scale), were determined from fits with k_{app} for phase 2 fixed to 26 s\(^{-1}\). Phase 1 data were fit to a hyperbola by nonlinear least means squares calculations by the Kaleidagraph program. For phase 1, k_{app} = 120 s\(^{-1}\) and K_{d, app} = 110 μM. Values of k_{obs} for phase 3 were also fit to a hyperbola with k_{app} = 14 s\(^{-1}\) and K_{d, app} = 270 μM, reduction of XO. All three phases in XO reduction were fit to hyperbolae with k_{app} values of 290, 250, and 69 s\(^{-1}\) for phases 1, 2, and 3, respectively. Values for K_{d, app} of 69, 62, and 69 μM for phases 1, 2, and 3, respectively, were also determined.

Reduction of Xanthine Oxidoreductase with 4-Hydroxypyrimidine
[2-2H]4-OH-P is used to determine the $K_{d,app}$ values. This observation is best expressed as a $D(k/K_d)$ effect (see "Materials and Methods"). The $D(k/K_d)$ values are $2.3 \pm 0.3$ for the first phase and $2.8 \pm 0.3$ for the third phase in the reductive half-reaction. In the second phase of reduction, the observed rate constant of 26 s$^{-1}$ does not change when [2-2H]4-OH-P is used as substrate, indicating that movement of the hydrogen atom originating from 4-OH-P does not at all limit the rate of this reaction. Since the value of 26 s$^{-1}$ observed in this phase is similar to $k_{cat}$ of 24 s$^{-1}$, it is of great interest to identify what is occurring in this reaction.

An interpretation of the spectral changes and observed kinetics described above is given in Scheme 1. The first kinetic phase is interpreted as binding of the first equivalent of substrate by XDH$_{ox}$, rapid oxidation of 4-OH-P to uracil with concomitant reduction of Mo(VI) to Mo(IV) to give XDH$_{2e}$ (Scheme 1, steps $k_1$ to $k_3$). The second reaction phase shows essentially no rate dependence on substrate concentration. This second phase is proposed to represent slow release of product from XDH$_{2e}$ ($k_4$), a rapid re-equilibration of electrons ($k_5$), and a second round of reduction ($k_6$). The assumption that the second reducing equivalent reacts similarly as the first was made to account for the observed formation of an XDH$_{4e}$ intermediate, and this supports mainly by simulations described below. The reaction in phase 3 converts XDH$_{4e}$ to XDH$_{6e}$ and is interpreted as involving binding of the third equivalent of 4-OH-P, redistribution of electrons in the XDH$_{4e}$-4-OH-P complex to yield a Mo(VI)/two-2Fe/2S$_{red}$/FADH$_2$ species, and oxidation of 4-OH-P to uracil along with reduction of Mo(VI) to Mo(IV) (Scheme 1, steps $k_{10}$ to $k_{12}$). Once the electrons in XDH$_{6e}$ redistribute to form Mo(VI) (step $k_{12}$), the iron and flavin cofactors are completely reduced, and no further reaction can be monitored by absorbance methods.

**Kinetics of XO Reductive Half-reaction—Time courses**, along with simulations to the data, at 450, 550, and 620 nm are shown for the reaction of 4.9 $\mu$M XO with 500 $\mu$M 4-OH-P (Fig. 3B). Data were recorded in the stopped flow spectrophotometer at individual wavelengths. All traces were fit to three exponentials with Program A. Although absorbance changes at 450 and 620 nm could be fit adequately to two exponentials, those at 550 nm clearly required three. These data at 500 $\mu$M 4-OH-P were fit to $k_{obs}$ values in order of appearance of 250, 250, and 64 s$^{-1}$. All reaction phases exhibit hyperbolic rate dependence on substrate concentration (Fig. 4B). Values for $k_{app}$ and $K_{app}$ for each phase are given in Table II. Reaction traces measured with [2-2H]4-OH-P as reductant were similar in form to those using the proteo-substrate (Fig. 5B). Kinetic deuterium isotope effects on the reductive half-reaction of XO are also given (Table II). The $k_{app}$ values for phases 1 and 2 each have kinetic isotope effects of 1.5 $\pm$ 0.2. All three phases demonstrate $D(k/K_d)$ effects with a range of 2.2 to 3.8.

A proposed model for the reductive half-reaction of XO with 4-OH-P is given in Scheme 2. Rapid binding of the first equivalent of substrate to oxidized enzyme (step $k_1$) is followed by rapid reduction of the molybdenum center of XO and oxidation of 4-OH-P to uracil, forming species C (step $k_2$). Since the spectra of intermediates 1 and 2 in Fig. 2B both correspond closest to two-electron-reduced forms of XO, the first two reduction phases must involve formation of two different XO$_{2e}$ species (D and E). Phase 1 has been assigned to transfer of one electron from Mo(IV) to a 2Fe/2S$_{red}$ center and corresponds to step $k_3$ in the reduction of XDH (Scheme 1). The isotope effect on this reaction is predicted to be an effect on protonation of molybdenum prior to electron transfer. The second phase in reduction has been assigned to further re-equilibration of electrons from molybdenum to 2Fe/2S and FAD chromophores. The
product uracil is proposed to be released in one of these two steps. There is little justification for an intrinsic isotope effect on this second electron-transfer reaction. The isotope effect on this second phase is most likely observed because phases 1 and 2 are poorly resolved. It is more accurate to consider species C in Scheme 2 decaying to species E at an overall rate of 140 ± 15 s⁻¹ with a kinetic isotope effect of 1.5 ± 0.2. Species D is known to exist from spectral data, but difficulty in resolving its rates of formation and decay preclude a more thorough model. The last phase in reduction of XO with 4-OH-P involves conversion of XO₂⁻ to XO₆ⁿ at an overall rate of 69 s⁻¹. To reduce XO by 4 more electrons, 2 additional eq of 4-OH-P must bind, reduce, and dissociate from the enzyme. These steps have been proposed in Scheme 2, but the paucity of information describing those reactions precludes proposing a model with more detail. It is most likely that product release and/or intramolecular electron transfer contribute to determining the rate in phase 3.

Reductive Half-reaction of XDH with Equimolar Substrate—To study the initial events when XDH is reduced by the first two-electron equivalents of substrate, XDH was reacted anaerobically in the stopped flow instrument with a stoichiometric concentration of 4-OH-P. An equimolar amount of substrate was used to maximize the amount of signal observed. It must be emphasized that this reaction is not performed under conditions of pseudo-first order kinetics. Spectra derived from model-free global analysis of diode array stopped flow data are presented for the reaction of 20 μM XDH reacted anaerobically with 20 μM 4-OH-P (Fig. 6A). One intermediate species is observed (spectrum 2, Fig. 6A) as XDH₁ sockfd is converted to XDH₂ sockfd. The intermediate is formed at a kobs of 23 s⁻¹. This intermediate corresponds to reduction of one 2Fe/2S center and presumably to oxidation of molybdenum from Mo(IV) to Mo(V). Its spectrum differs distinctly from the first intermediate in the full reduction experiment in the extent of 2Fe/2S reduction and in fact more closely resembles that of the first intermediate in the reduction of XO by excess substrate (Fig. 2B). This new intermediate with only one 2Fe/2S center reduced presumably occurs in the full reduction experiment but is not resolved due to similarity in rate and spectral changes of formation of the individual 2Fe/2S reduced species. Attempts to detect an intermediate with only one 2Fe/2S center reduced by reacting XDH with excess (500 μM) 4-OH-P at 4 °C failed to resolve this species (not shown). The second phase of the XDH equimolar reduction reaction at kobs of 27 s⁻¹ results in further bleaching of 2Fe/2S absorbance up to 1.27 electrons on the two 2Fe/2S centers as well as the formation of 0.62 eq of FADH₂ as seen by the increase in long wavelength absorbance (spectrum 3, Fig. 6A). This spectrum is completely consistent with that of XDH₂ sockfd from equilibrium experiments (15).

Single wavelength experiments were also performed with 1 eq of 4-OH-P, and representative reactions at final concentrations of 20 μM XDH and 20 μM 4-OH-P are shown at 450 and 550 nm (Fig. 6B). The biphasic behavior of the reaction is...
Reduction of Xanthine Oxidoreductase with 4-Hydroxypyrimidine

XDH and XO, each at 40 μM active enzyme, were each mixed anaerobically with an equal volume of 40 μM 4-OH-P in a diode array stopped flow instrument. Reactions were performed at 25 °C, pH 7.5. Singular value decomposition of the data with the Specfit program indicated that three species are present in the XDH reaction and that two species are involved in the XO reaction. Global analysis of the data yielded model-free spectra. A, spectrum 1: spectra for oxidized XDH, spectrum 2, an intermediate XDH species; spectrum 3, the final partially reduced XDH. The final spectrum of partially reduced XO is also shown in spectrum 4. A, inset, a plot of \( k_{\text{obs}} \) versus enzyme and substrate. Phase 1 of the XDH reaction fits to a hyperbola with \( k_{\text{app}} = 96 \pm 7 \text{ s}^{-1} \) and a concentration of 46 ± 3 μM (Fig. 6A, inset). Equimolar reduction with [2-2H]4-OH-P shows similar behavior with \( k_{\text{app}} \) of \( 43 \pm 6 \text{ s}^{-1} \) and a half-maximal concentration of 44 ± 10 μM. There is a kinetic isotope effect of 2.3 ± 0.2 on the rate of reduction.

Similar behavior is observed when XDH is reduced by 1 eq of [2-2H]4-OH-P (not shown). The extrapolated rate constant of the concentration-dependent step (phase 1) is 23 ± 5 s⁻¹, with a half-maximal rate at 21 μM, when [2-2H]4-OH-P is substrate. This deuterium kinetic isotope effect of 1.7 ± 0.4 agrees well with those of 1.7 and 1.6 observed in the full reduction experiment. Observed rates for phase two with [2-2H]4-OH-P as substrate have a fair amount of scatter, yet average at a concentration-independent rate constant of 21 ± 4 s⁻¹ (not shown).

Reductive Half-reaction of XO with Equimolar Substrate—A solution of 20 μM active XO was reacted anaerobically with 20 μM 4-OH-P (final concentrations) in the diode array instrument. Global analysis of the resulting spectra with the Specfit program indicated that only two species, and no intermediates, were present in the reaction. Diode array data were fit to a \( k_{\text{obs}} \) of 40 s⁻¹. A spectrum is presented (spectrum 4, Fig. 6A) of XO at the end of the reaction. This spectrum has 0.19 electrons on 2Fe/2S, 1.2 electrons on FAD as FADH₂, and no significant amount of FADH, an electron distribution similar to that predicted for XO₂⁻ at equilibrium (16).

Reactions at a final concentration of 15 μM XO and 15 μM 4-OH-P are shown by the dashed curves in Fig. 6B. A decrease in absorbance occurs at \( k_{\text{obs}} \) of 24 s⁻¹. The slow absorbance increase seen at 450 nm varies between experiments in rate and extent of reaction. This has been interpreted as reoxidation by contaminating O₂. Varying the concentration of both XO and 4-OH-P results in hyperbolic dependence of \( k_{\text{obs}} \) on reactant concentration, with \( k_{\text{app}} \) of 96 ± 7 s⁻¹ and a maximal concentration of 46 ± 3 μM (Fig. 6A, inset). Equimolar reduction with [2-2H]4-OH-P shows similar behavior with \( k_{\text{app}} \) of 43 ± 6 s⁻¹ and a half-maximal concentration of 44 ± 10 μM. There is a kinetic isotope effect of 2.3 ± 0.2 on the rate of reduction. Results from these equimolar reduction experiments are consistent with reactions \( k₁-k₄ \) in Scheme 2. The value of 96 s⁻¹ is lower than the expected 140 s⁻¹ from Scheme 2 possibly because the exponential fitting used is more appropriate for reactions performed under pseudo-first order conditions. Simulations of the reaction between equimolar substrate and enzyme according to the mechanism and constants used in Scheme 2 recapitulated the observed results (not shown), indicating that the extrapolated rate constant of 96 s⁻¹ in these equimolar reductions is not inconsistent with the model in Scheme 2.

Reductive Half-reaction with Excess Substrate and Excess Product—The product, uracil, is expected to bind in the same site on XDH as the substrate 4-OH-P. Only reactions that involve substrate binding will be inhibited by uracil, as product dissociation reactions will only be affected in extent and not rate of reaction. When XDH is reduced by 300 μM 4-OH-P in the presence of 5 mM uracil, the reaction traces have the same shape as those observed with 4-OH-P alone, and only the first and third phases are slowed (data not shown). Under the conditions described above, phase one is decreased from 89 to 59 s⁻¹ and phase three is slowed from 7.7 to 3.2 s⁻¹. By contrast, the rate of the phase 2 reaction only varies from 23 to 25 s⁻¹ when uracil is added. This observation is consistent with the interpretation of the first and third phase in reduction occurring after a previous binding reaction and the second phase involving product release. At least 5 mM uracil is required to significantly slow the observed rate of phases one and three, indicating that uracil binds much weaker to XDH than does 4-OH-P.

When XO was reduced by 300 μM 4-OH-P, in the presence of 5 mM uracil, the observed rate constants of the first and second phases were reduced from 240 to 160 s⁻¹ for phase 1 and from

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![Graph](image-url)
240 to 120 s$^{-1}$ for phase 2, compared with the reaction with 300 μM 4-OH-P alone. The rate of the third phase was essentially unaltered, with a rate of 52 s$^{-1}$ with 4-OH-P alone and 48 s$^{-1}$ with 4-OH-P and uracil. This insensitivity of the third phase of XO reduction to uracil concentration is unexpected due to the observation of substrate concentration dependence in this reaction. This suggests that product release and/or intramolecular electron transfer events determine the rate in phase 3.

Simulation of XDH Data—The six-electron reduction of XO or XDH by 4-OH-P is clearly a complex reaction, involving three separate events of substrate binding, reduction of Mo(VI) to Mo(IV), product dissociation, and electron redistribution among the four distinct redox centers of the enzymes. Despite this complexity, however, reduction is observed to occur in three distinct phases at all wavelengths, i.e. the observed rate constants must be composite ones containing the appropriate microscopic rate constants for the reaction steps involved. Therefore the mechanism of the reductive half-reaction of XDH with 4-OH-P presented in Scheme 1 has been simulated in its entirety using Program A. A rapid equilibrium was assumed for the binding reactions in steps $k_1$, $k_6$, and $k_{10}$, and all other reactions were assumed to be irreversible. The first order reactions in steps $k_2$-$k_2$, $k_6$-$k_6$, and $k_8$-$k_8$ were each simplified to a single step. Simulated traces of the reaction of XO with 500 μM 4-OH-P closely approximate the actual data (Fig. 3A), as do values of the apparent rate and dissociation constants determined by fitting simulations at various substrate concentrations (Fig. 4A). Analysis of simulated traces resulted in the following kinetic parameters: $k_{1,app} = 130$ s$^{-1}$, $K_{d,app} = 110$ μM, $k_{2,app} = 24$ s$^{-1}$, $k_{3,app} = 13$ s$^{-1}$, $K_{d,app} = 300$ μM. Rate and dissociation constants used in the simulations are described in the legend to Fig. 3 and are taken from Table II with the exceptions discussed below. Extinction coefficients used in the simulations were calculated from theoretical electron distributions at various redox states and from the analysis of single wavelength stopped flow data.

By varying the initial concentration of 4-OH-P in the simulation, a family of curves was generated demonstrating the concentration dependence of the reaction. Analysis of these theoretical traces by Program A yielded values very close to the kinetic parameters determined experimentally (Fig. 3). An exception to this is the value of $k_{app}$ for phase one in the full reduction experiment. A rate constant of 180 s$^{-1}$ was used for $k_2$ and $k_6$ in Scheme 1 to yield a $k_{app}$ value near 120 s$^{-1}$ for the first phase in analysis of simulated curves. Given a mechanism as complex as that discussed here, it is expected that individual steps may be poorly resolved. The mechanism described in Scheme 1 involves 12 steps with 4 involving absorbance changes, yet the simulations are fit adequately with only three apparent rate constants. Also, better agreement was achieved by setting the true $K_d$ for steps $k_2$, $k_6$, and $k_8$ in Scheme 1 to 85 μM, instead of the observed $K_{d,app}$ of 110 μM. As the $K_d$ determined kinetically is only an apparent dissociation constant (see discussion under “Materials and Methods”), this difference is insignificant. Steps $k_2$-$k_2$, $k_6$-$k_6$, and $k_8$-$k_8$ in Scheme 1 are a repetition of steps $k_1$-$k_1$. The constants given in Scheme 1 reflect values determined from simulations to the data. Isotope effects given in Scheme 1 were calculated from rate constants derived from simulations of [2-3H]- and [2-1H]-4-OH-P data and agree well with observed isotope effects.

The equimolar reduction experiments were successfully simulated using a truncated version of Scheme 1 which ended with species F. Simulations of full and equimolar reduction experiments with [2-3H]- and [2-1H]4-OH-P as substrate also gave results consistent with the actual data (not shown). These simulations demonstrate that the mechanism of XDH reduction presented here is consistent with all of the experimental data; however, no amount of simulations can establish the veracity or uniqueness of a mechanism.

Simulation of XO Data—The model presented in Scheme 2 for the reductive half-reaction of XO with 4-OH-P was also simulated. The binding reactions in steps $k_{1,app}$ and $k_{2,app}$ were made rapid equilibria. Steps $k_2$ and $k_6$ were combined as one reaction. Simulation of reaction traces at 500 μM 4-OH-P closely approximate the actual data (Fig. 3B). Rate constants used were those in Table II. Extinction coefficients used, described in the legend to Fig. 3, were determined from electron distributions at various redox states and from the spectra in Fig. 2B. The initial concentration of 4-OH-P was varied in the simulations to determine changes in $k_{obs}$ (Fig. 4B). Analysis of such curves yielded values in excellent agreement with the constants in Table II. All three phases showed hyperbolic rate dependence on 4-OH-P, supporting the model in Scheme 2 in which the reactions of phases 1 and 2 both exhibit saturation kinetics even though they follow the same substrate binding reaction. Analysis of these simulations resulted in kinetic parameters within 10% of the experimentally determined values. Isotope effects given in Scheme 2 were calculated from rate and dissociation constants determined from simulations and agree well with the experimentally determined values. Reactions in which XO was reduced with a single equivalent of 4-OH-P were also successfully simulated according to Scheme 2 (not shown).

Oxidative Half-reaction of XO—Since $k_{cat}$ for XO turnover of 4-OH-P is 32 s$^{-1}$ at 25 °C and pH 7.5, and since the slowest rate of reduction of XO is 69 s$^{-1}$ under the same conditions, the reaction of fully reduced XO with O$_2$ was studied to determine the extent to which the oxidative half-reaction of XO limits catalysis. XO that had been reduced with sodium dithionite was reacted in the stopped flow instrument with various concentrations of O$_2$ (Fig. 7). Identical results were obtained with XO that had been reduced with titanium(III) citrate and with XO that had been photo-reduced. All absorbance traces were fit to three exponentials. Consistent with previous reports under different conditions (30–32), the reaction involves three phases as follows: a poorly resolved lag phase, a phase whose rate is hyperbolically dependent on O$_2$ concentration and involves the majority of the spectral changes, and a phase whose rate is...
linearily dependent on O₂ concentration and involves a minor portion of the absorbance changes (Fig. 7, inset). Observed rate constants were averaged between the three experiments that used the three different reductants. Values of $k_{obs}$ for the lag phase varied chaotically from 26 to 88 s⁻¹ such that no reliable rate constant could be extracted (not shown). The extrapolated apparent rate constant at infinite O₂ concentration for the second phase was the same at all wavelengths, 64 ± 8 s⁻¹, although the $K_{d,app}$ values at 450 and 550 nm were quite different, 360 ± 50 and 130 ± 30 μM, respectively. While $K_{d,app}$ values for oxygen binding are reported, it is likely that oxygen actually reacts in a purely second order fashion, as has been shown with XDH (13, 26), and that saturation kinetics are observed due to some other first order process, such as intramolecular electron transfer or protonation/deprotonation reactions. The total absorbance change at the end of the second phase corresponds to further oxidation of FAD and one 2Fe/2S center. The last phase involves net oxidation of one 2Fe/2S center in a bimolecular reaction with a rate constant of 1.5 ± 0.3 × 10⁴ M⁻¹ s⁻¹ (Fig. 7, inset). It should be emphasized, however, that the last phase presumably is mediated by reaction of O₂ with reduced flavin species since reduced, deflavo XO is oxidized extremely slowly by O₂ (3). From these rate constants it can clearly be seen that the oxidative half-reaction at 25 °C, pH 7.5, certainly limits $k_{cat}$ for 4-OH-P/oxygen turnover to a significant extent.

**DISCUSSION**

The results presented here for the reductive half-reaction of bovine milk XDH with 4-OH-P as reducing substrate are similar to those obtained with xanthine as substrate (22). Both reactions involve the same enzyme intermediates, but the substrate concentration dependence of the reaction with 4-OH-P provides significantly more information. XDH reacts with the first equivalent of xanthine in a multi-step process involving two observable concentration-independent steps as follows: 1 electron transfer from molybdenum to an 2Fe/2S center at 15 s⁻¹, and product release and further electron transfer from molybdenum to 2Fe/2S centers at 13 s⁻¹ (22). With 4-OH-P as substrate the first phase exhibits saturation kinetics with a first order rate constant of 180 s⁻¹ and a true dissociation constant of 85 μM. The spectral changes accompanying this reaction are very similar to those with xanthine as substrate. That the first one-electron transfer (with product bound) is increased from 15 s⁻¹ with urate to 180 s⁻¹ with uracil demonstrates that electron re-equilibration is very sensitive to the nature of the bound ligand. The second step observed with 4-OH-P as substrate occurs at 26 s⁻¹ and is attributed to product release. The spectrum at the end of this phase is indistinguishable between the xanthine and 4-OH-P reactions.

Although the third phase in xanthine reduction of XDH exhibits inverse concentration dependence on $k_{obs}$ due to non-productive binding of xanthine, the same phase with 4-OH-P as substrate exhibits hyperbolic concentration dependence on $k_{obs}$ presumably due to the much higher $K_{d}$ of 4-OH-P. Product dissociation at 26 s⁻¹ limits the rate of reduction and is numerically equivalent to $k_{cat}$ at 24 s⁻¹ with NAD as oxidizing substrate, within the error of the data. The rate of product release is therefore increased modestly from 13 s⁻¹ for urate to 26 s⁻¹ for uracil.

Results from the current study of the reductive half-reaction of XO with 4-OH-P differ from previous results with xanthine in the much higher rates of reduction and looser substrate binding observed. As previous experiments with other substrates were performed at pH 8.5, and the work reported here is at pH 7.5, comparison of absolute rate and dissociation constants cannot be made strictly. An important result from experiments with XO and 4-OH-P is the resolution of species D in Scheme 2. This first intermediate observed when XO is reduced by excess 4-OH-P has the same absorbance spectrum as the intermediate measured when XDH is reduced by 1 eq of 4-OH-P. These results support the conclusion that with both XDH and XO, the reaction with the first equivalent of 4-OH-P involves the same one 2Fe/2Sred:Mo(V) intermediate and that the absorbance changes observed for the second intermediate in the experiments with excess substrate are a function of the different redox potentials of XDH and XO cofactors. Note that the possibility that the one-electron on iron-sulfur in the first intermediate is shared between the two 2Fe/2S centers cannot be ruled out from these experiments. However, with 1-methyl-xanthine or at pH 10 with xanthine, the $g = 2.12$ EPR signal of 2Fe/2S II could clearly be observed prior to the $g = 1.90$ signal of 2Fe/2S I (33). Although the current study gives no indication on which 2Fe/2S the one-electron may reside, the 2Fe/2S center nearest the molybdenum seems most reasonable as the electron distribution appears to be under kinetic control. From electron nuclear double resonance experiments (34), and the *Desulfovibrio gigas* aldehyde oxoreductase structure (35), Lowe and colleagues suggest the 2Fe/2S center nearer the molybdenum to be 2Fe/2S II. Assignment of a rate-limiting step in XO reduction by 4-OH-P is complicated by the observation of no XOred intermediate. Such an intermediate must exist and is presumably not observed due to a similarity in rate and absorbance changes for formation and decay of the XOred species. Likely events that could contribute to the $k_{app}$ value of 69 s⁻¹ observed are intramolecular electron transfer and product release.

In reacting with the first equivalent of substrate, the molybdenum center undergoes a complete single turnover. It is possible that two different steps in the reductive half-reaction could be sensitive to deuterium substitution in the reducing substrate: transfer of the 2-H of 4-OH-P to the molybdenum center on reducing Mo(VI) to Mo(IV) and subsequent transfer of that proton from molybdenum to solvent on re-oxidation of molybdenum. Isotope effects on both steps are proposed to be observed; the former is measured as a $^{2H}$/$^{1H}$ (k/k') effect, and the latter is seen as a $^{2H}$/$^{1H}$ effect. Rapid quench studies with xanthine as substrate indicate that 1 eq of urate is formed from xanthine within the dead time (5–10 ms) of a rapid quench instrument for both bovine milk XO and chicken liver XDH (36). The primary reduction step can therefore be estimated to occur at least as fast as 800 s⁻¹ for these enzymes. The first equivalent of uracil is expected to be formed from 4-OH-P at least as quickly as from xanthine. As deuterium substitution should have no effect on substrate binding, the increase in $K_{d,app}$ values with both XDH and XO can best be explained in terms of an intrinsic isotope effect upon the oxidation of 4-OH-P, a reaction prior to the first irreversible step. The isotope effect on this step is best expressed as a $^{2H}$/$^{1H}$ (k/k') effect that ranges from 1.9 to 2.3 in the reaction with the first equivalent of substrate. This is entirely analogous to a $^{13}V$/13K isotope effect, as discussed in steady-state kinetics (see “Materials and Methods”). In the present study, a large isotope effect is presumed to exist on $k_{cat}$ in Schemes 1 and 2. This isotope effect is masked by the slower, irreversible step that follows, $k_{cat}$. As transferring an electron from Mo(V) to the higher potential 2Fe/2S II center involves a change in redox potential of 95 mV (15, 16), step $k_{cat}$ in Schemes 1 and 2 may be considered essentially irreversible. Such a model is consistent with the observed change in $K_{d,app}$ on deuterium substitution. The kinetic isotope effect for the intramolecular electron transfer reaction, $k_{cat}$, is proposed to be due to deprotonation of the molybdenum center immediately prior to (and possibly controlling) one-electron transfer.
oxidation. Loss of the Mo-SH proton, derived from xanthine, on oxidation from Mo(IV) to Mo(V) has been proposed from EPR studies (18, 37). The magnitude of the kinetic isotope effect on the rate of 2Fe/2S reduction, $k_d$, of 1.5 with XO and 1.6 with XDH, is close to the kinetic deuterium isotope effects ranging from 1.3 to 2.2 observed by monitoring the Mo(V) EPR spectra of XO in rapid freezing experiments (33). Xanthine and 1-methyloxanthine were used as substrates in those studies at pH 8.5. Deprotonation of reduced molybdenum as the isotopically sensitive step is consistent with the observation of similar kinetic isotope effects with different substrates. The first and second phases in XO reduction each have kinetic isotope effects of 1.5 ± 0.2. These rate constants are poorly resolved, however, and it is most likely that only one of these steps is isotopically sensitive. The value of 1.5 is close to that of 1.6 observed in the XDH reaction. This similarity in isotope effect between XDH and XO implies the existence of similar transition states for the reaction of both enzyme forms with the first equivalent of substrate.

The models proposed in Schemes 1 and 2 provide a simple explanation for the steady-state deuterium isotope effects in XDH turnover. The ${{k}^{'}{V}/K_{4-OH, p}}$ value of 1.7 is due to an isotopically sensitive step, substrate oxidation, $k_d$, or electron redistribution, $k_R$, that precedes the rate-limiting step in reduction, product release, $k_p$. The ${{k}^{'}{V}} = 1.0$ is consistent with this model. The pattern of lines in the Lineweaver-Burk plot for xanthine/NAD turnover of XDH suggests that the enzyme follows a ternary complex mechanism at high substrate concentration, and a ping-pong mechanism at low substrate concentration. A similar change in mechanism has been observed in the oxygen concentration dependence of l-amino acid oxidase turnover (38). Further evidence in support of a ternary complex comes from pre-steady-state data. The reductive half-reaction with 4-OH-P is limited in rate by uracil release at 26 s$^{-1}$. The slowest step in the oxidative half-reaction with NAD is approximately 42 s$^{-1}$. A classical ping-pong mechanism predicts $k_{cat}$ to be $(26 s^{-1})(42 s^{-1})/(26 + 42 s^{-1}) = 16 s^{-1}$, significantly below the 24 s$^{-1}$ measured. Since the rates of reduction and oxidation are each quite close to $k_{cat}$, a ternary complex mechanism may be operative at high substrate concentrations. This is quite reasonable considering that the sites of enzyme reduction (molybdenum) and oxidation (FAD) are physically removed.

The steady-state kinetic deuterium isotope effects for XO can be explained as with XDH. The slowest apparent rate constant observed in the reductive half-reaction of XO with 4-OH-P has a value of 69 s$^{-1}$ but is certainly a complex function of rates involved in the conversion of XO$_{6e}$ to XO$_{6o}$ XO. The majority of the absorbance change in the oxidative half-reaction of XO at 25 °C and pH 7.5 was determined to occur with an apparent rate constant of 64 s$^{-1}$. Based on a simple ping-pong mechanism, using 69 s$^{-1}$ for $k_{red}$ and 64 s$^{-1}$ for $k_{ox}$ provides an adequate explanation of $k_{cat}$, (69 s$^{-1}$)(64 s$^{-1})/(69+64 s^{-1}) = 33 s^{-1}$, in excellent agreement with the measured value of 32 s$^{-1}$.

While the individual steps in reduction of XDH are more clearly resolved than in XO, the overall mechanisms of reduction are quite similar. Most differences in reduction of the enzyme forms are due to the stabilization of different reduced chromophores, consistent with the changes in the redox potentials of the FAD center (15, 16). Therefore, the conformational changes that have such a pronounced effect on the nature of the FAD moiety seem to have little influence on the molybdopterin center. The actual absorbance changes observed on reduction correspond to electron transfer events from molybdenu to the other centers of the enzyme. Measurement of these intramolecular electron transfer reactions in XO by pH jump (5), flash photolysis (6), and pulse radiolysis (7) studies have yielded rates from 100 to 330 s$^{-1}$ near neutral pH. These rates are much faster than catalysis, consistent with the rapid equilibrium hypothesis of Olson et al. (4). Rates of reduction observed in the current study (180 s$^{-1}$ with XDH and 290 s$^{-1}$ with XO) are consistent with those measuring intramolecular electron transfer alone. The observation of a deuterium kinetic isotope effect on some of these electron transfer events is consistent with the prototropic control of electron transfer proposed by Hille (39). With both XO and XDH, the first intermediate detected corresponds to a single reduced 2Fe/2S center and presumably Mo(V) (species D in Schemes 1 and 2). This is not a thermodynamically stable species as the redox potentials of both enzyme forms predict two-electron-reduced enzyme to have the majority of electrons in the iron and flavin centers (15, 16). This reduction of a 2Fe/2S center is proposed to be the initial step in a discrete path of electron transfer from molybdenum to flavin. Differences in the rates at which this 2Fe/2S center is reduced between XO and XDH may reflect differences in the distance or orientation between the molybdopterin and 2Fe/2S centers. The contribution of these factors cannot be evaluated between XO and XDH in the absence of structural data. In the reaction of both XO and XDH with the first equivalent of substrate, intramolecular transfer of the second electron from the molybdenum center appears to be triggered by product release. Following product release, electrons re-equilibrate rapidly according to the relative redox potentials of the centers. Such “ligand gating” of electrons has also been observed in other enzymes containing multiple redox centers such as protocatechuate dioxygenase reductase from Pseudomonas cepacia (40) and trimethylamine dehydrogenase from bacterium W$_2$A$_1$ (41) and appears to be a general phenomenon.

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