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Articles

Electron Transfer within Xanthine Oxidase: A Solvent Kinetic Isotope Effect Study†

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ABSTRACT: Solvent kinetic isotope effect studies of electron transfer within xanthine oxidase have been performed, using a stopped-flow pH-jump technique to perturb the distribution of reducing equivalents within partially reduced enzyme and follow the kinetics of reequilibration spectrophotometrically. It is found that the rate constant for electron transfer between the flavin and one of the iron–sulfur centers of the enzyme observed when the pH is jumped from 10 to 6 decreases from 173 to 25 s^{-1} on going from H_2O to D_2O, giving an observed solvent kinetic isotope effect of 6.9. An effect of comparable magnitude is observed for the pH jump in the opposite direction, the rate constant decreasing from 395 to 56 s^{-1}. The solvent kinetic isotope effect on k_{obs} is found to be directly proportional to the mole fraction of D_2O in the reaction mix for the pH jump in each direction, consistent with the effect arising from a single exchangeable proton. Calculations of the microscopic rate constants for electron transfer between the flavin and the iron–sulfur center indicate that the intrinsic solvent kinetic isotope effect for electron transfer from the neutral flavin semiquinone to the iron–sulfur center designated Fe/S I is substantially greater than for electron transfer in the opposite direction and that the observed solvent kinetic isotope effect is a weighted average of the intrinsic isotope effects for the forward and reverse microscopic electron-transfer steps. In both H_2O and D_2O the preponderance of the kinetic effect of a change in the thermodynamic driving force for the intramolecular electron-transfer reaction is on the microscopic rate constant for electron transfer from the flavin semiquinone to the iron–sulfur center. The results emphasize the importance of prototropic equilibria in the kinetic as well as thermodynamic behavior of xanthine oxidase and indicate that protonation/deprotonation of the isoalloxazine ring is concomitant with electron transfer in the xanthine oxidase system.

Xanthine oxidase is a complex metalloflavoprotein containing a molybdenum center, two 2Fe/2S centers of the spinach ferredoxin variety, and flavin adenine dinucleotide in each of its two identical and independent subunits (Hille & Massey, 1985; Bray, 1988). The reductive half-reaction of the catalytic cycle (xanthine hydroxylation to form uric acid) takes place at the molybdenum center of the enzyme (Bray et al., 1964), and the oxidative half-reaction (dioxygen reduction to peroxide or superoxide, depending on the level of enzyme reduction; Hille & Massey, 1981; Porras et al., 1981) at the FAD (Komai et al., 1965). Intramolecular electron transfer from the molybdenum to the FAD is thus an integral aspect of catalysis, and the enzyme serves a useful system in which to examine biological electron transfer. The prevailing hypothesis describing the behavior of xanthine oxidase is the rapid equilibrium model proposed by Olson et al. (1974), the basic premise of which is that reducing equivalents in partially reduced xanthine oxidase distribute themselves among the four redox-active centers of the enzyme according to their relative

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reduction potentials and do so on a time scale that is rapid relative to turnover. The model has been extremely effective in predicting a variety of thermodynamic and kinetic properties of both native xanthine oxidase (Olson et al., 1974; Cammack et al., 1976) and enzyme containing chemically modified flavin derivatives of varying reduction potentials (Hille et al., 1981; Hille, 1987). The validity of the assumption that electron transfer within xanthine oxidase is rapid has been tested by flash photolysis (Bhattacharyya et al., 1983; Edmondson et al., 1987), pH-jump (Hille & Massey, 1986), and pulse radiolysis (Anderson et al., 1986; Hille & Anderson, 1991) techniques. While there is not complete agreement with regard to the specific rate constants for electron transfer between specific pairs of centers as determined by flash photolysis on the one hand, and pulse radiolysis and pH jump on the other, there is general agreement that the observed rates are substantially faster than turnover under the experimental conditions.

Both the quinone/semiquinone and semiquinone/hydroquinone half-potentials of the flavin site of xanthine oxidase exhibit a strong pH dependence, from which a pK of 9.5 for the prototropic equilibrium associated with the semiquinone oxidation state has been determined from potentiometric data (Porras & Palmer, 1982). By contrast, the reduction potentials for the iron–sulfur centers of the enzyme exhibit a much weaker pH dependence. Thus, in partially reduced xanthine oxidase, iron–sulfur reduction is thermodynamically preferred at high pH while at low pH reduction of the flavin to either the semiquinone or hydroquinone predominates. The distribution of reducing equivalents within partially reduced xanthine oxidase can thus be perturbed by changes in pH in a manner that is quantitatively attributable to the known pH dependence of the reduction potentials for the several centers in the enzyme (Porras & Palmer, 1982). With a pH-jump technique to perturb the oxidation–reduction equilibrium within partially reduced xanthine oxidase (following the reequilibration spectrophotometrically), it has been shown that electrons equilibrate rapidly between the FAD and one of the iron–sulfur centers of xanthine oxidase (assigned Fe/S I on the basis of calculations of the electron distribution within the partially reduced enzyme from the relative reduction potentials) with k_{obs} = 160 s^{-1} at pH 6 and 330 s^{-1} at pH 8.5 (Hille & Massey, 1986). The redox-linked prototropic equilibrium at an exchangeable site on the flavin demonstrated in these experiments raises the question of the influence of protonation/deprotonation events on the observed kinetics. In order to gain further insight into the role of prototropic equilibria in the kinetics of electron transfer events within xanthine oxidase, the solvent kinetic isotope effect on electron transfer has been examined by pH-jump stopped-flow experiments. The results demonstrate a large solvent kinetic isotope effect arising from a single exchangeable proton and are interpreted as indicating that proton uptake/loss is concomitant with electron uptake/loss at the flavin site of xanthine oxidase.

Experimental Procedures

Xanthine oxidase was prepared by the method of Massey et al. (1969), with the exception that unpasteurized milk rather than the buttermilk from pasteurized cream was utilized as the starting material. A CM-52 column was used to remove contaminating lactoperoxidase as the final step in the preparation (Morrison & Hultquist, 1963). In order to ensure a uniform enzyme population in the experiments described herein, the enzyme used in the present study was converted to the so-called desulfo form by reaction with 0.1 M sodium cyanide for 1.5 h at 5 °C (Massey & Edmondson, 1970). This reaction results in the replacement of a catalytically essential sulfido ligand of the active center molybdenum with an oxo group, releasing one equivalent of thiocyanate, but does not affect the properties of the FAD and iron–sulfur centers of the enzyme in any way. On the basis of calculations of electron distributions within partially reduced enzyme at both high and low pH, the inactivation reaction has the desirable effect of increasing the change in electron distribution between FAD and iron–sulfur in the pH-jump experiment. Use of the desulfo form of xanthine oxidase in the present studies also eliminates possible experimental complications due to the interaction of bisulfite (formed in the course of enzyme reduction by sodium dithionite) with the molybdenum center of functional enzyme (Fish et al., 1990). It is to be emphasized that the process being monitored kinetically in the present study, the equilibration of reducing equivalents between the flavin and iron–sulfur centers of the enzyme, does not directly involve the molybdenum center.

Stopped-flow experiments were carried out with a Kinetic Instruments device interfaced to a Zenith 158 personal computer. The apparatus was modified by isolating the light source on an optical breadboard from the pneumatic drive in order to minimize vibration artifacts. Data acquisition and analysis was performed with software from On-Line Instruments Systems. The anaerobic glassware used in the stopped-flow experiments consisted of a tonometer fitted with a sidearm cuvette and a ground glass joint through which a gas-tight syringe could be fitted for the incremental addition of dithionite stock solutions. UV/visible spectrophotometry was carried out with a Hewlett-Packard 8452A diode-array spectrophotometer interfaced to an HP 9153C personal computer. Anaerobic reductive titrations were carried out in anaerobic cuvettes fitted with gas-tight syringes that contained concentrated sodium dithionite stock solutions in the appropriate buffer and solvent isotope. The reductive titration data, presented as proportionality plots of the fractional absorbance change at 550 nm versus that at 450 nm, were simulated as described previously (Olson et al., 1974; Hille et al., 1981), by using a program written in BASIC. The extinction changes associated with reduction (relative to oxidized enzyme on a per molybdenum basis) used for the two iron–sulfur centers in these simulation were $\Delta\epsilon_{450}$ = −7.2 mM^{-1} cm^{-1}, $\Delta\epsilon_{550}$ = −3.45 mM^{-1} cm^{-1} (Hille et al., 1984). The extinction changes used for $FADH_2$ were $\Delta\epsilon_{450}$ = −12.2 mM^{-1} cm^{-1} and $\Delta\epsilon_{550}$ = 0 mM^{-1} cm^{-1} (Olson et al., 1974); for $FADH^{\bullet}$ the values used were $\Delta\epsilon_{450}$ = −9.0 mM^{-1} cm^{-1} and $\Delta\epsilon_{550}$ = +4.4 mM^{-1} cm^{-1}. These latter values were estimates taken from the spectrophotometric data for the neutral flavin semiquinone of glucose oxidase (Massey & Hemmerich, 1980) and flavodoxin (Dubourdieu & LeGall, 1970). It was found empirically that the simulations at pH/D 8.5 and 10 were not particularly sensitive to moderate (~20 mV) changes in the difference between the two flavin half-potentials, owing to the substantially greater reduction potential for the $FADH^{\bullet}/FADH_2$ couple relative to that for the $FAD/FADH^{\bullet}$ couple (which results in little accumulation of flavin semiquinone over the course of the reductive titration in this pH range). By contrast to the insensitivity to the separation in flavin half-potentials, the simulations were found to be quite sensitive to the flavin midpoint potential used in the simulations, consistent with previous work (Hille et al., 1981). Similarly, the simulations were quite sensitive to the separation of the two iron–sulfur potentials, a greater separation giving rise to a greater inflection in the proportionality plots. Because of the large accumulation of the neutral flavin semiquinone in the course of reductive ti-

Table I: Relative Reduction Potentials Used in the Simulation of Reductive Titrations of Xanthine Oxidase at pH/D 6.0, 8.5, and 10.0[a]

	pH 6.0	pD 6.0	pH 8.5	pD 8.5	pH 10.0	pD 10.0
Fe/S $I_{ox/red}$	−0.09	−0.09	−0.09	−0.09	−0.09	−0.09
Fe/S $II_{ox/red}$	0.0	0.0	0.0	0.0	0.0	0.0
FAD/FADH$^{\bullet}$	0.04	0.09	−0.08	−0.06	−0.14	−0.10
FADH$^{\bullet}$/$FADH_2$	0.06	0.08	0.0	0.02	−0.02	0.02
FAD/$FADH_2$[b]	0.05	0.085	−0.04	−0.02	−0.08	−0.04
Mo^{VI}/Mo^{V}	−0.08	−0.08	−0.14	−0.14	−0.14	−0.14
Mo^{V}/Mo^{IV}	−0.14	−0.14	−0.12	−0.12	−0.12	−0.12

[a] The values used for simulation of data at pH 8.5 and 10.0 were derived from the work of Porras and Palmer (1982), all others were those values that gave the best fits to the data, as described in the text. It is to be noted that neither flavin half-potential exhibits a 60 mV/pH unit dependence owing to the ionization of the N_5 proton of the neutral semiquinone (pK = 9.5) and N_1 of the hydroquinone (pK 7.5) of the isoalloxazine ring [cf. Porras and Palmer (1982)]. [b] The flavin midpoint potential (i.e., the average of the potentials for the FAD/FADH$^{\bullet}$ and FADH$^{\bullet}$/$FADH_2$ couple at each experimental condition).

trations at low pH and its appreciable extinction coefficient at 550 nm (which results in a net *increase* in absorbance above 500 nm for approximately the first third of the reductive titration at low pH), computer simulations of the proportionality plots of data obtained at pH 6.0 proved to be quite sensitive to both the midpoint potential of the flavin and the separation of the two half-potentials. As pointed out previously (Hille et al., 1981), simulations of the type described here are completely insensitive to the reduction potentials assigned to the Mo(VI/V) and Mo(V/IV) couples, since the molybdenum center does not contribute significantly to the absorbance changes at 450 and 550 nm observed upon reduction of xanthine oxidase. The relative reduction potentials that gave the best fits to the data at pH/D 6.0, 8.5, and 10.0 are given in Table I. For the simulations of the data obtained at pH 8.5 and 10, these relative values were in agreement with the potentiometric data of Porras and Palmer (1981). At pH 6.0, however, a 70 mV more positive potential for the flavin was required than that obtained from the potentiometric data in order to obtain a reasonable fit to the data. Given the technical difficulties in the potentiometric experiment at low pH cited by Porras and Palmer (1981), we regard the higher relative potential for the flavin to be more reliable.

Potentiometric titrations of free FAD were carried out in an anaerobic titration vessel using sodium dithionite as reductant. Silver/silver chloride and platinum electrodes were used, and the poised system potential measured with an Orion Research 701 digital volmeter. The electrochemical system was standardized against ferro-/ferricyanide to obtain potentials relative to the standard hydrogen electrode.

D_2O (99.9% enriched) was obtained from Cambridge Isotopes Laboratory. Isotopic mixtures of enzyme and/or buffers were obtained by mixing appropriate volumes of separately prepared solutions in H_2O and D_2O, with the latter prepared taking into account the necessary correction of the pH meter reading to obtain the pD (pD = meter reading + 0.4; Gutfreund, 1977). Solutions of D_2O were prepared immediately before use and kept in capped vessels to prevent contamination with atmospheric moisture over the course of the experiments. Enzyme in D_2O was obtained by passage through a Sephadex G-25 column equilibrated with D_2O buffer (hydration of the dried gel material was also carried out with D_2O). Enzyme solutions for the pH-jump experiments were made anaerobic by periodically flushing with dry O_2-scrubbed argon and partially reduced with a concentrated sodium dithionite stock solution prior to being mounted on the stopped-flow apparatus for the pH-jump experiment. While it has previously been shown that the observed rate of electron equilibration within xanthine oxidase is independent of the level of enzyme reduction over a wide range (Hille & Massey, 1986), care was taken to ensure the level of reduction for each enzyme sample used was close to 50% (as monitored by the enzyme absorbance change at 450 nm observed upon reduction). Previous studies have also shown that, under the present experimental conditions, the final pH after mixing is within 0.10 pH units of the more concentrated buffer prior to mixing (Hille & Massey, 1986).

Sodium dithionite was from Virginia Chemicals, and sodium cyanide was from Aldrich. MES[1] [2-(N-morpholino)-ethanesulfonic acid], EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid], and CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffers were from Sigma. All other reagents were of the highest purity commercially available and used without further purification.

Results

Solvent Kinetic Isotope Effect on Electron Transfer within Xanthine Oxidase. It has been shown previously that the distribution of reducing equivalents within partially reduced xanthine oxidase is pH dependent and quantitatively consistent with the known pH dependence of the relative reduction potentials of the enzyme (Porras & Palmer, 1982). Six equivalents are required for full reduction: two at the molybdenum center in going from Mo^{VI} to Mo^{IV}, two at the flavin, and one at each of the iron–sulfur centers. In enzyme reduced to the level of three electron equivalents per enzyme (i.e., half reduced), low pH favors relatively greater formation of the neutral flavin semiquinone and Mo^{V}, whereas high pH favors relatively greater reduction of the iron–sulfur center designated Fe/S I (Hille & Massey, 1986). At pH 10 only approximately 2% of the flavin is in the semiquinone oxidation state, and most of this (approximately 75% of the semiquinone population or slightly more than 1% of the total enzyme flavin) is in the deprotonated anionic form (pK ~ 9.5; Porras & Palmer, 1982). By contrast, at pH 6 approximately 12% of the total enzyme flavin is present as the flavin semiquinone, entirely as the protonated neutral form. For a pH 10 to 6 jump, therefore, a net transfer of reducing equivalents from reduced Fe/S I to oxidized flavin to give the neutral flavin semiquione takes place with a prominent absorbance increase observed at 525 nm due to both formation of the flavin semiquinone and oxidation of Fe/S I (Hille & Massey, 1986).[2] On the basis of the extinction coefficients of the centers involved and the fact that approximately 10 times as much flavin is involved in the electron-transfer reaction as in the direct protonation of anionic semiquinone, the spectral change due to direct

[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; ENDOR, electron–nuclear double resonance; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid.

[2] The effect of the pH jump is thus to perturb the distribution of reducing equivalents between the flavin and molybdenum center, on the one hand, and Fe/S I on the other. Only equilibration between the neutral flavin semiquinone and iron–sulfur center can be observed on the stopped-flow time scale, it having been demonstrated that equilibration between the molybdenum and iron–sulfur centers takes place too rapidly to be seen (Hille & Anderson, 1991). The observed stopped-flow kinetics in a pH-jump experiment thus reflect an approach to a new equilibrium position between the two centers, and the observed rate constant is the sum of the rate constants for electron transfer from the neutral flavin semiquinone to oxidized Fe/S I and the reverse.

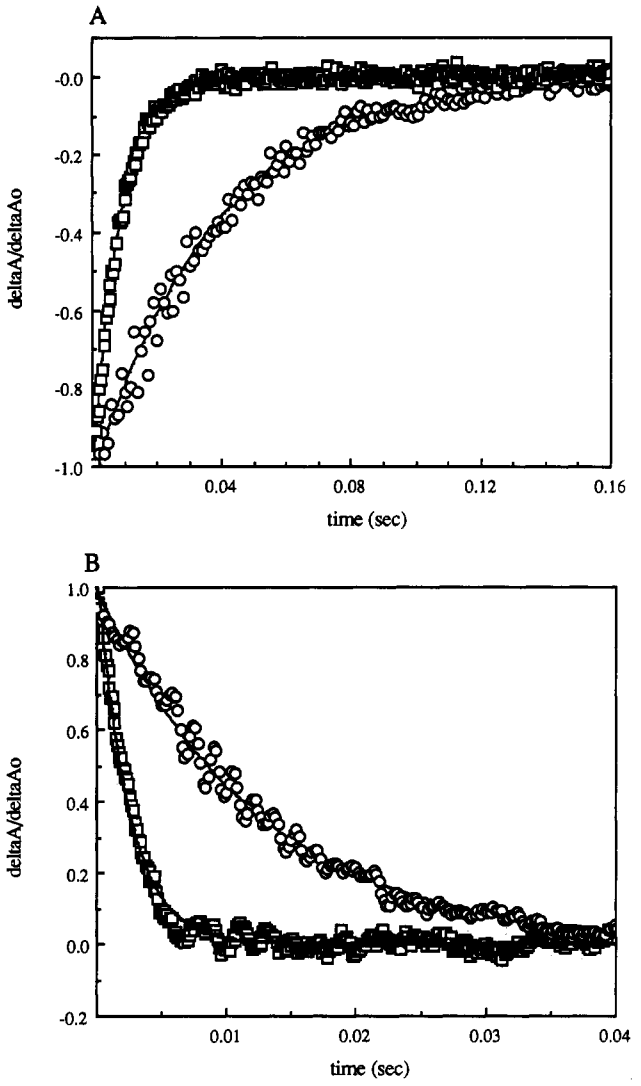

FIGURE 1: Kinetic transients observed at 525 nm for pH-jump experiments in H_2O (squares) and D_2O (circles). (A) Partially reduced xanthine oxidase (79 μM) in 10 mM CAPS pH (or pD) 10.0 was mixed with an anaerobic solution of 0.1 M MES and 0.1 N KCl, pH (or pD) 6.0. Enzyme reduction was carried out by using a concentrated solution of sodium dithionite to give approximately 50% of the spectral change at 450 nm upon reduction. The solid lines represent computer fits to the data in H_2O and D_2O of 173 and 27 s^{-1}, respectively. (B) Partially reduced xanthine oxidase (79 μM) in 10 mM MES and 0.1 N KCl, pH (or pD) 6.0 was mixed with an anaerobic solution of 0.1 M CAPS, pH (or pD) 10.0. The solid lines represent computer fits to the data in H_2O and D_2O of 395 and 56 s^{-1}, respectively.

protonation of anionic semiquinone present is expected to be no more than 5–10% of the total absorbance change seen at 525 nm in the pH 10–6 jump experiment and has not been observed experimentally.

It is clear from the above discussion that, in a pH 10–6 jump experiment, formation of the neutral flavin semiquinone necessarily involves protonation as well as uptake of one electron equivalent. The reverse is true for a pH 6–10 jump, with a net loss of a proton as well as an electron by the flavin site (to form the oxidized flavin quinone). In order to assess the solvent kinetic isotope effect on electron transfer within xanthine oxidase, pH-jump experiments of the type done previously were performed in H_2O and D_2O. Figure 1A shows the effect of going from H_2O to D_2O on the kinetics observed when partially reduced desulfo xanthine oxidase in 10 mM CAPS and 0.1 N KCl, pH/D 10 is mixed with anaerobic 0.1 M MES and 0.1 N KCl, pH/D 6.0. The observed rate constants of 173 ± 5 and 25 ± 2 s^{-1} in H_2O and D_2O, respectively,

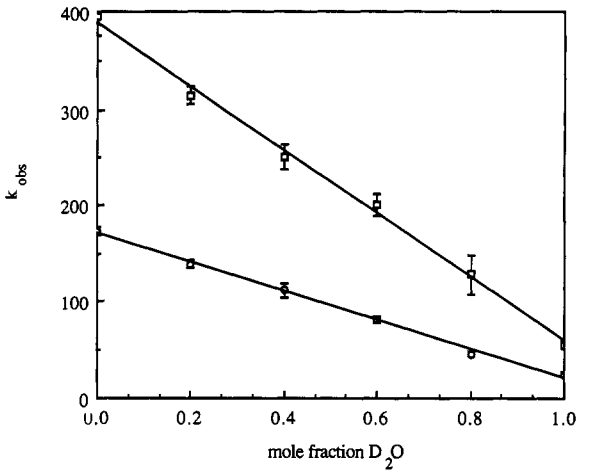

FIGURE 2: Dependence of the observed rate constant in the pH jump experiment on the mole fraction of D_2O in the reaction mix. (Circles) pH 10→6 jump; (squares) pH 6→10 jump. The data reflect the average of three to five separate kinetic transients, with the standard deviation in each case indicated by the error bar. The linear relationship observed for the pH jump in each direction is indicative of the effect arising from a single exchangeable proton coupled to the electron equilibration event. Enzyme concentrations at each mole fraction D_2O were approximately 80 μM.

give an observed solvent kinetic solvent isotope effect of 6.92, consistent with the involvement of exchangeable protons in the reequilibration of reducing equivalents within the enzyme. The value of 173 s^{-1} in H_2O is in good agreement with the value of 160 s^{-1} obtained in previous studies using enzyme that was approximately 75% active (Hille & Massey, 1986) and supports the interpretation that the observed kinetics does not involve the molybdenum center of the enzyme. Figure 1B shows the results of a pH-jump experiment in the opposite direction, with partially reduced enzyme initially in 10 mM MES and 0.1 N KCl, pH 6.0, being mixed anaerobically with 0.1 M CAPS and 0.1 N KCl, pH 10. The observed rate constants in H_2O and D_2O are 395 ± 19 and 56 ± 4 s^{-1}, respectively, indicating a solvent kinetic isotope effect of 7.05, identical with that observed in the pH 10→6 experiment, within experimental error.

Proton Inventory of the Solvent Kinetic Isotope Effect on Electron Transfer. In order to ascertain whether the observed solvent kinetic isotope effect is due to one or more exchangeable protons in the reaction, a proton inventory experiment was undertaken, in which the solvent kinetic isotope effect at varying mole fractions of D_2O was determined (Klinman, 1978). In such an experiment, a linear relationship is expected for the involvement of a single exchangeable proton, a quadratic relationship for two, a cubic relationship for three, etc. The results are shown in Figure 2 for both the pH/D jump from pH 10 to 6 and the reverse. It is seen that the observed solvent kinetic isotope effect exhibits a linear dependence on the mole fraction of D_2O for the pH jump in each direction. The experimental error in the kinetic data is within the limits required to resolve a one-proton process from a two-proton one (Schowen, 1978). Kresge (1973) has pointed out that it is possible under special circumstances to obtain straight lines in proton inventory experiments for systems that involve multiple protons. This requires a large number of exchangeable sites exhibiting secondary kinetic isotope effects, each of which influences the curvature of the solvent inventory plot in the opposite sense that a second primary proton site does. This is statistically unlikely in the present case, since it must arbitrarily be assumed that each of the secondary sites influences the curvature of the proton inventory plot in the

same direction. Further, there is only a single obvious candidate for the ionization responsible for the primary effect, namely the redox-linked proton associated with the semiquinone oxidation state. We thus regard it as unlikely that an appreciable amount of the observed solvent kinetic isotope effect is due to some unknown number of secondary protons and conclude that the observed effect is due principally to the redox-linked proton of the flavin semiquinone.

Solvent Isotope Effect on the Relative Reduction Potentials of the Flavin and Iron–Sulfur Centers of Xanthine Oxidase. It has been demonstrated for a number of model systems that reduction potentials change on going from H_2O to D_2O (Weaver & Nettles, 1980; Guarr et al., 1983). Theory predicts that such a change in thermodynamic driving force should have an effect on the observed electron transfer kinetics (Marcus, 1965, 1968). In order to ascertain the magnitude of the solvent isotope effect on the thermodynamics of electron transfer between flavin and iron–sulfur centers in xanthine oxidase, reductive spectrophotometric titrations of desulfo xanthine oxidase in H_2O and D_2O were carried out to ascertain any change in the relative reduction potentials of the flavin and iron–sulfur centers of xanthine oxidase. A plot of the fractional absorbance change at 450 nm (where the flavin and iron–sulfur centers contribute approximately equally to the absorbance change observed upon reduction of the enzyme) versus that at 550 nm (where the iron–sulfur centers are responsible for the entirety of the spectral change) provides an accurate indication of the relative potentials of the centers. A positive deflection from proportionality indicates a more positive value for the average of the iron–sulfur center potentials relative to the flavin midpoint potential and a negative deflection the reverse. Such proportionality plots can be computer-simulated by using the known extinction changes at the relevant wavelengths for each of the centers and a set of relative reduction potentials (Olson et al., 1974), and it has been demonstrated that the technique is able to resolve changes as small as 10 mV in the relative reduction potentials of the redox-active centers of xanthine oxidase (Hille et al., 1981). Such an analysis of the data is particularly useful from the standpoint of the present application in that the technique is sensitive only to changes in the relative reduction potentials of the several centers and not their values relative to an external standard, since it is the relative potentials that determine the thermodynamic driving force for intramolecular electron transfer between specific pairs of sites in the enzyme. The necessity of directly determining the reduction potentials of the various centers of the enzyme is thereby obviated.

Figure 3 shows the results of reductive titrations with desulfo xanthine oxidase at pH/D 6.0, 8.5, and 10.0, along with simulations to the data with the relative reduction potentials given in Table I and extinction coefficients given under Experimental Procedures. It is noteworthy that the results in 0.1 M EPPS, pH 8.5, and 0.1 M CAPS, pH 10.0, are in good quantitative agreement with previous results obtained in pyrophosphate buffer at pH 8.5 (Olson et al., 1974) and 10.2 (Hille et al., 1981), respectively. The results of this analysis are consistent with an increase in the flavin midpoint potential (i.e., the average of the increase in potentials for the FAD/FADH$^\bullet$ and FADH$^\bullet$/$FADH_2$ couples) of approximately 40, 20, and 35 mV on going from H_2O to D_2O at pH/D 10, 8.5, and 6.0, respectively. These values are obtained from a comparison of the average of the two flavin half-potentials (relative to the iron–sulfur centers of the enzyme) in H_2O and D_2O at any given pH/D (Table I). The increase in flavin reduction potential on going from H_2O to D_2O was confirmed in direct

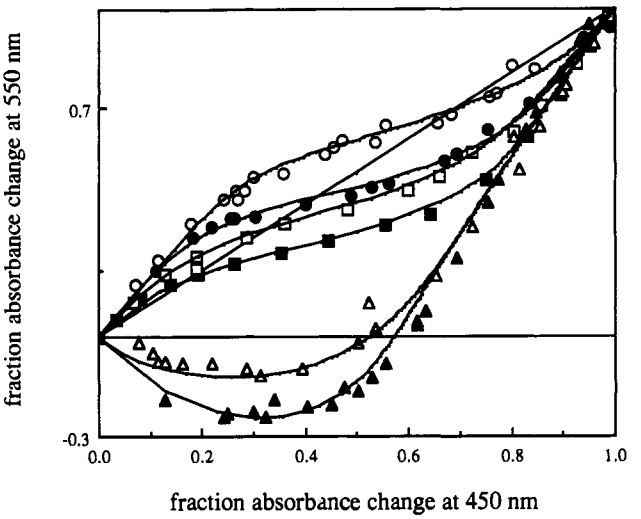

FIGURE 3: Plots of the fractional absorbance change at 550 nm versus that at 450 nm observed in the course of reductive titration of xanthine oxidase as a function of pH. The data are for enzyme in H_2O (open symbols) of D_2O (closed symbols) at the following buffer conditions: 0.1 M CAPS and 0.1 N KCl, pH/D 10.0 (circles); 0.1 M EPPS and 0.1 N KCl, pH/D 8.5 (squares); and 0.1 M MES and 0.1 N KCl, pH/D 6.0 (triangles). Enzyme concentrations were on the order of 50 μM in the reductive titrations. The lines are simulations of the data performed as described under Experimental Procedures. The results are consistent with increases in the flavin midpoint potential of 40, 20, and 35 mV at pH/D 10.0, 8.5, and 6.0, respectively, on going from H_2O to D_2O (see Table I).

potentiometric titrations of free flavin adenine dinucleotide in 0.1 M EPPS and 0.1 N KCl, pH 7.0, where it was found that the midpoint potential of the free cofactor increased by approximately 40 mV on going from H_2O to D_2O as solvent (data not shown). The data are consistent with the N_1–D and N_5–D bonds of reduced flavin being more stable than the corresponding N–H bonds.

Discussion

The present pH-jump results demonstrate that electron transfer between one of the iron–sulfur centers of xanthine oxidase (presumably that designated Fe/S I, see below) and the enzyme's flavin exhibits an extremely large observed solvent kinetic isotope effect of 7 for both the pH 10→6 and 6→10 jumps. On the basis of the proton inventory experiments, it appears that the observed effect is due to a single prototropic equilibrium associated with the flavin semiquinone. This interpretation is consistent with the known pH dependence of the flavin reduction potentials (Porras & Palmer, 1982), and the observation that one-electron reduction of the flavin of xanthine oxidase involves proton uptake to give the neutral semiquinone at pH 8.5 or below. The magnitude of the effect is comparable to that seen in primary C–H bond cleavage reactions where bond breaking is rate limiting and clearly indicates that the redox-linked proton at the flavin site of xanthine oxidase is in motion in the course of the electron transfer reaction moving through the transition state. The magnitude of the observed solvent kinetic isotope effect in the present studies emphasizes the extent to which protonation/deprotonation of the flavin site of xanthine oxidase is linked to its oxidation–reduction behavior and has a kinetic as well as thermodynamic manifestation. It is undoubtedly relevant from a physiological standpoint since the extremes in pH used in the present study serve only to perturb the oxidation–reduction equilibrium in the present experiments. The pK for the flavin semiquinone of xanthine oxidase, ~9.5, is such that even at neutral pH the protonated neutral flavin semiquinone

will be formed upon one-electron reduction of the flavin at neutral pH (with protonation as a necessary consequence).

Solvent kinetic isotope effects due entirely to solvation are typically in the range of 1.2–1.5, and rarely exceed 4 even in those cases where protonation/deprotonation events are associated with catalysis [in, e.g., the trypsin-catalyzed hydrolysis of peptide bonds (Schowen, 1978)]. The solvent kinetic isotope effect on electron transfer in simple inorganic systems has been summarized (Weaver & Nettles, 1980), with values in the range 1.0–2.6 cited. A notable exception is electron transfer between $[Ru^{II}(bpy)_2(py)OH_2]^{2+}$ with $[Ru^{IV}(bpy)_2(py)O]^{2+}$, yielding $2[Ru^{III}(bpy)_2(py)OH]^{2+}$. The solvent kinetic isotope effect observed for this reaction is 16.1 and exhibits a linear dependence on the mole fraction of D_2O (Binstead et al., 1981). The suggestion was made that the system may represent the limiting case of hydrogen atom rather than independent electron/proton transfer. Hydrogen atom transfer per se is not a possibility in the present case, however, and the proton taken up by the flavin upon reduction to the semiquinone must come from elsewhere than the iron–sulfur center that is the electron donor, either from solvent or a solvent-exchangeable site on the protein.

Relatively few studies of solvent kinetic isotope effects on electron transfer in biological systems have been reported. McCray and Kihara (1979) determined a solvent kinetic isotope effect of 1.4 for the oxidation of ferrocytochrome *c* by ferricyanide and noted that this was comparable to the effect observed for the reduction of the photosynthetic reaction center by its cytochrome (Kihara & McCray, 1973). Using an extremely precise difference technique, Okamura and Feher (1986) have determined a small reverse solvent kinetic isotope effect on 0.94 for the charge recombination reaction in the photosynthetic reaction center of *Rhodopseudomonas sphaeroides*. Interestingly, it was possible to assign the protons responsible for the effect to the exchangeable sites hydrogen-bonded to the $Q_A^{\bullet-}$ donor in the reaction (on the basis of a correlation of the rate of exchange at these sites, as monitored by ENDOR spectroscopy, and the onset of the kinetic isotope effect). More relevant to the present results, electron transfer from the flavin semiquinone of $NADP^+$-bound ferredoxin reductase to oxidized ferredoxin has been found to decrease from 55 to 12 s^{-1} on going from H_2O to D_2O (Batie & Kamin, 1984), although these workers note that in all likelihood ferredoxin dissociation is rate limiting under the experimental conditions. The solvent kinetic isotope effect on electron transfer has also been examined in the stable complex of adrenodoxin with adrenodoxin reductase (Lambeth, 1982), another system that contains both a flavin and an iron–sulfur center. In this case, intramolecular electron transfer from the semiquinone of the NADPH-complexed reductase to adrenodoxin, monitored indirectly by subsequent cytochrome *c* reduction at the reduced iron–sulfur center, exhibits an isotope effect in the range of 1.5–1.8. The effect is observed only when the flavin semiquinone is the electron donor and only in the presence of NADPH. Finally, Beckmann and Frerman (1985) have reported a solvent effect of comparable magnitude for the rate of comproportionation of electron-transferring flavoprotein (catalyzed by ETF:Q_1 oxidoreductase). Again, however, the measurement of electron transfer was indirect and involved multiple turnover of the system. In each of the above cases involving electron transfer between a flavin site and an iron–sulfur center, the experimental system is either complicated by reversible protein–protein association events and/or indirect measurements of electron transfer between the sites. The present results thus constitute the first direct examination of the solvent kinetic isotope effect on intramolecular electron transfer in a flavin-containing system.

Thorpe and co-workers (Mizzer & Thorpe, 1981; Lehman & Thorpe, 1990) have considered the role of redox-linked prototropic equilibria associated with the flavin ring in attempting to understand the effect of exogenous ligands on the kinetic and thermodynamic behavior of medium chain acyl-CoA dehydrogenase. These workers note that the flavin must frequently change ionization state as a result of electron transfer into or out of the isoalloxazine ring so as to avoid the formation of thermodynamically unfavorable species and that this might plausibly affect the kinetics of the system. For example, the flavin semiquinone anion should be a more effective electron donor than the neutral form since the latter must deprotonate to avoid formation of the energetically unfavorable oxidized flavin cation ($pK \sim 0$). Similarly, the hydroquinone anion $FADH^-$ should be more a more effective electron donor than the neutral $FADH_2$, and the protonated neutral semiquionone should be a more effective electron acceptor than the semiquinone anion (since reduction of the latter would give rise to the extremely unfavorable dianionic hydroquinone). This reasoning provides an explanation for the paradox that exogenous ligands to reduced acyl-CoA dehydrogenase that tend to stabilize anionic forms of the flavin semiquinone and hydroquinone [rendering the enzyme a thermodynamically poorer reductant by raising its reduction potential (Gorelick et al., 1985; Lenn et al., 1990)] also accelerate the kinetics of enzyme reoxidation. This is true with either the physiological electron acceptor electron-transferring flavoprotein or inorganic reagents such as ferricinium salts, the observed rate constant increasing by as much as 600-fold [for the oxidation of the semiquinone of the acyl-CoA dehydrogenase by 200 μM ferricinium ion (Lehman & Thorpe, 1990)]. Large solvent kinetic isotope effects would reasonably be expected with protonation/deprotonation occurring either concomitantly with electron transfer or with rate-limiting protonation/deprotonation occurring prior or subsequent to electron transfer. The results of the pH 6→10 jump experiment with xanthine oxidase reported here are consistent with both of these possibilities. The pH 10→6 jump experiment is, however, rather more problematic from the standpoint of rate-limiting protonation/deprotonation. Since the flavin becomes reduced and protonated in the course of the pH jump in this direction, rate-limiting protonation of the anion semiquinone must be invoked in order to account for the large observed solvent kinetic isotope effect. However, the rate of protonation of the radiolytically generated anionic semiquinone of *Megasphaera elsdenii* flavodoxin, for example, has been determined to be quite fast, 2.6×10^5 s^{-1} at pH 6.0 (Anderson et al., 1987), and the neutral flavin semiquinone is known to be formed rapidly in pulse radiolysis experiments with xanthine oxidase as well (Anderson et al., 1986; Hille & Anderson, 1991). Thus in the case of xanthine oxidase the solvent kinetic isotope data are most consistent with an electron transfer mechanism wherein protonation/deprotonation is concomitant with electron transfer. By concomitant, it is meant that at the instant electron transfer occurs the N_5–H bond is either partially broken (for electron transfer from $FADH^\bullet$ to the oxidized iron–sulfur center) or formed (for electron transfer from the reduced iron–sulfur center to oxidized FAD). Such a situation does not violate the Franck–Condon principle but simply indicates that in the course of passage through the electron-transfer transition state the hydrogen atom must be in motion (albeit slowly relative to the electron transfer event), thereby giving rise to the observed kinetic isotope effect.

Assuming concomitant electron transfer and protonation/deprotonation, the pH-jump-driven electron transfer can be treated as a single-step equilibrium:

$$\begin{bmatrix} \text{FAD} \\ \text{Fe/S}_{red} \end{bmatrix} \quad H^+ \rightleftharpoons \quad \begin{bmatrix} \text{FADH}^\bullet \\ \text{Fe/S}_{ox} \end{bmatrix}$$

This permits the solvent kinetic isotope effect on the individual microscopic rate constants for the forward and reverse steps in the equilibration to be determined. On the basis of calculations of the distribution of reducing equivalents within xanthine oxidase containing three electrons per molybdenum (i.e., reduced by 50%), it is clear that the equilibration being perturbed in the present experiments is that between the iron–sulfur center designated Fe/S I and the flavin FAD/FADH• couple (Hille & Massey, 1986). Within the context of a single-step mechanism, the ratio of the rate constants for forward and reverse electron transfer for this equilibrium is given by the equilibrium constant (calculated in turn from the relative reduction potentials using the Nernst equation) at each condition, while the observed rate constant in the pH-jump experiment is the sum of the microscopic rate constants for the forward and reverse steps. With two equations and two unknowns, it is possible to determine the rate constants associated with electron transfer from the flavin to Fe/S I, and the reverse. The results of such calculations explicitly take into account the change in thermodynamic driving force of the electron-transfer reaction upon going from H_2O to D_2O and are given in Table II. Despite the relatively large uncertainties inherent in such calculations (owing principally to the fact that the values for the microscopic rate constants are particularly sensitive to the values of the relative reduction potentials used to calculate the equilibrium constant for the overall reaction when the equilibrium constant is small and to small changes in the magnitude to the equilibrium constant when it is large), several observations are noteworthy. First, the rate constant for electron transfer from Fe/S I to FAD is much less sensitive to the change in thermodynamic driving force observed on going from a final pH of 6 to 10 in both H_2O (172 vs 93 s^{-1}) and D_2O (25 vs 28 s^{-1}) than is electron transfer in the opposite direction (0.72 vs 301 s^{-1} in H_2O and 0.02 vs 28 s^{-1} in D_2O). Further, the solvent kinetic isotope effect for electron transfer from the flavin neutral semiquinone to oxidized Fe/S I is appreciably larger than that for electron transfer in the opposite direction.[3] While there is a large uncertainty in the value for the solvent kinetic isotope effect on electron transfer from FADH• to oxidized Fe/S I at pH 6 (because of the extremely small values calculated for this step relative to k_{obs} for the oxidation–reduction equilibration arising from the large value for the equilibrium constant), this is not the case for the kinetic parameters calculated at pH 10, and we conclude that the kinetic isotope effect on electron transfer from the neutral flavin semiquinone to Fe/S I is in excess of 10. This value is extremely large but is not without chemical precedent [e.g., the ruthenium system cited above; Binstead et al. (1981)] and may reflect the greater need to deprotonation the neutral flavin semiquinone in the case of xanthine oxidase so as to avoid formation of the oxidized flavin cation. Together, these observations indicate that there is a substantial kinetic barrier to electron transfer out of the neutral flavin semiquinone that arises from the necessity of cleaving the N_5–H bond. Finally, it is to be noted that the observed solvent kinetic isotope effect for the equilibration at both pH/D values is the weighted sum of the solvent effects on the forward and reverse microscopic electron transfer steps, as should be the case.

Table II: Thermodynamic and Kinetic Parameters for Electron Transfer between the Flavin and Fe/S I of Xanthine Oxidase[a]

	$\delta(\Delta E)$ (mV)	K_{eq}	k_{obs}	$k_{flavin \rightarrow Fe/S}$	$k_{Fe/S \rightarrow flavin}$
pH 6.0	+130	160	173 s^{-1}	1.0 s^{-1}	172 s^{-1}
pD 6.0	+180	1120	25 s^{-1}	0.02 s^{-1}	24.9 s^{-1}
SIE	+50	7.0	6.9	(50)	6.9
pH 10.0	−50	0.14	395 s^{-1}	346 s^{-1}	49 s^{-1}
pD 10.0	−10	0.68	56 s^{-1}	33 s^{-1}	23 s^{-1}
SIE	+40	4.9	7.0	10.5	2.1

[a] $\delta(\Delta E)$ is the difference in reduction potentials from Table I for the FAD/FADH• and Fe/S $I_{ox/red}$ couples at the various conditions. K_{eq} is the equilibrium constant for the oxidation–reduction equilibrium: FAD + H^+ + Fe/s I_{red} ⇌ FADH• + Fe/s I_{ox}. SIE is solvent isotope effect.

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Registry No. FAD, 146-14-5; neutral FADH semiquinone, 81138-30-9; xanthine oxidase, 9002-17-9; deuterium, 7782-39-0.

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[3] The effect is even more pronounced when the solvent isotope effect on each microscopic rate constant is corrected for the change in thermodynamic driving force. Using the equation $k_H/k_D \sim (K^H/K^D)^{1/2}$ (Weaver & Nettles, 1980; Marcus, 1965, 1968) and the equilibrium constant changes given in Table II, it is possible to estimate the amount of the isotope effect on each microscopic rate constant that arises simply from the increase in the FAD/FADH• potential in going from H_2O to D_2O. Correction factors of 2.6 at pH/D 6 and 2.2 at pH/D 10 are obtained. For the Fe/S → FAD electron transfer, the calculated solvent isotope effect must be divided by this correction factor, since the reaction becomes more favorable in D_2O and a portion of the kinetic isotope effect is thus thermodynamic in origin. The correction reduces the kinetic solvent isotope effect on the Fe/S → FAD electron transfer rate constant to 2.7 at pH/D 6 and abolishes it altogether at pH/D 10. There is a corresponding increase in the values for the solvent isotope effect on the FAD → Fe/S electron transfer rate constant, giving rise to extremely large numbers. This is possibly a reflection of a tunneling mechanism for electron transfer in xanthine oxidase (at least for electron transfer involving the flavin), but given the several approximations and assumptions made in these calculations it is impossible to justify a firm conclusion at present. It is nevertheless clear that the solvent isotope effect on electron transfer from the neutral flavin semiquinone to Fe/S I is the principal source of the observed solvent effect on the oxidation–reduction equilibration monitored in the pH-jump experiments described here.

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