Electron Transfer in Milk Xanthine Oxidase as Studied by Pulse Radiolysis*

Russ Hille† and Robert F. Anderson§

From the §Department of Medical Biochemistry, Ohio State University, Columbus, Ohio 43210 and the †Gray Laboratory of the Cancer Research Campaign, P. O. Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, United Kingdom

Electron transfer within milk xanthine oxidase has been examined by the technique of pulse radiolysis. Radiolytically generated N-methylnicotinamide radical or 5-deazalumiflavin radical has been used to study electron transfer within the enzyme so that subsequent equilibration among the four redox-active centers of the enzyme (a molybdenum center, two iron-sulfur centers, and FAD) could be monitored spectrophotometrically. Experiments have been performed at pH 6 and 8.5, and a comprehensive scheme describing electron equilibration within the enzyme at both pH values has been developed. All rate constants ascribed to equilibration between specific pairs of centers in the enzyme are found to be rapid relative to enzyme turnover under the same conditions. Electron equilibration between the molybdenum center and one of the iron-sulfur centers of the enzyme (tentatively assigned Fe/S I) is particularly rapid, with a pH-independent first-order rate constant of approximately 8.5 \times 10^4 \text{ s}^{-1}. The results unambiguously demonstrate the role of the iron-sulfur centers of xanthine oxidase in mediating electron transfer between the molybdenum and flavin centers of the enzyme.

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Xanthine oxidase is a complex metalloflavoprotein containing in each of its two identical and noninteracting subunits one molybdenum center, two iron-sulfur centers of the 2Fe/2S spinach ferredoxin variety and flavin adenine dinucleotide (1, 2). The reductive half-reaction (oxidation of xanthine to form uric acid) takes place at the molybdenum center of the enzyme and the oxidative half-reaction (dioxynogen reduction to form peroxide or superoxide, depending on the level of enzyme reduction) at the flavin (3, 4). Internal electron transfer between the molybdenum and flavin centers of the enzyme, presumably mediated by the iron-sulfur centers, is thus an integral aspect of the catalytic cycle, and xanthine oxidase serves as an extremely useful system in which to examine biological electron transfer. Electron transfer within xanthine oxidase has been examined previously by flash photolysis (5, 6), stopped-flow pH jump (7) and pulse radiolysis (8) techniques, with conflicting results sometimes being obtained. In the initial study utilizing the photolytically generated radical of 5-deazalumiflavin (5), it was concluded that reducing equivalents moved from one site in the enzyme to another only rather slowly (12–80 \text{ s}^{-1}, depending upon the specific pair of centers involved; Ref. 5), at rates comparable to catalysis itself (15 \text{ s}^{-1} in 0.1 \text{ M pyrophosphate, pH 8.5; Ref. 9}). Experiments utilizing the pH jump technique to rapidly perturb the distribution of reducing equivalents within partially reduced xanthine oxidase, on the other hand, indicated that over a wide range in pH the slowest rate at which reducing equivalents re-equilibrated between a specific pair of centers within the enzyme (the flavin and the iron-sulfur center designated Fe/S I) was a factor of 10 or greater than k_{cat} under the same experimental conditions (7). Equilibration between Fe/S I and the molybdenum center was found in these studies to be too fast to be quantitated by stopped-flow techniques (k_{eq} > 500 \text{ s}^{-1}). More recently, the flash photolysis work has been re-examined to indicate that electron transfer within xanthine oxidase is in fact rapid relative to turnover (6), although the rate constants obtained in this re-analysis were not entirely in agreement with those determined in the pH jump study (100 \text{ s}^{-1} for equilibration between the iron-sulfur center designated Fe/S II at pH 8.3, 25 °C, compared with a value of 200 \text{ s}^{-1} at pH 8.5, 20 °C, determined by pulse radiolysis; Ref. 8). We present here the results of further pulse radiolysis studies of the rates of electron transfer within xanthine oxidase utilizing the radiolytically generated radicals of N-methylnicotinamide and 5-deazalumiflavin as proximal reductants for the enzyme.

**MATERIALS AND METHODS**

Xanthine oxidase was prepared by the method of Massey et al. (10) with the addition of a CM52 column chromatography step at the end to remove contaminating lactoperoxidase (11). Functional enzyme was separated from the nonfunctional form (lacking the catalytically essential sulfido group of the molybdenum center) by the affinity chromatography procedure of Nishino et al. (12). When necessary, xanthine oxidase was quantitatively converted to the inactive desulfur form of the enzyme by reaction of enzyme with 0.1 M sodium cyanide for 1 h (13) followed by molecular sieve chromatography to remove unreacted cyanide. In the course of the previous pulse radiolysis work (8), it was found necessary to block a redox-active disulfide of xanthine oxidase (14) by prior reaction of dithionite-reduced enzyme with iodoacetate to prevent the reaction of radiolytically generated reducing equivalents with the intact disulfide. This was found to be the case in the present work as well, and is consistent with the observation of radiolytically reducible disulfide bonds in other proteins (15, 16). All experiments described here were performed with xanthine oxidase in which this disulfide had been blocked by reaction with iodoacetate so as to render it redox-inert (14).

Pulse radiolysis experiments were performed with the Van de Graaff accelerator system of the Gray Laboratory, Cancer Research Campaign, United Kingdom, described previously (8). Electron pulses were typically on the order of 100 ns, delivering doses that were varied over the range 2–30 grays. Dosimetry was performed using potassium thiocyanate (17) in order to quantitate the radiolytic yield of reducing equivalents under the experimental conditions. All reactions were performed with N_{2}O saturated solutions in the presence of...
0.1 M formate in order to convert the primary radiolytic radicals (e\text{aq}−, HO−, H\text{(H}_2\text{O}, H_2, HO^+)\) to CO_2, which in turn reduced the species used as the proximal reductant of the enzyme (either N-methyl nicotinamide or 5-deazaluminflavin). The concentrations and second-order rate constants for the overall reaction sequence, shown below, were such that under the experimental conditions the primary radical yield (0.68 μM/gray) was quantitatively converted to the proximal radical. In this way nonspecific recombination of the primary radiolytic radicals with the enzyme was avoided.

\[
\begin{align*}
H_2O & \rightarrow e_{\text{aq}}^- + HO^- + H^+ + NO_3^- + H_2O \rightarrow N_2 + HO^- \\
HO^- + NO_3^- + H^+ & \rightarrow N_2 + HO^- \\
CO_2^+ + \text{NMN}'(dF1) & \rightarrow CO_2 + \text{NMN}'(dF1)
\end{align*}
\]

It is possible that it was necessary to explicitly take into account the dimerization of the N-methyl nicotinamide radical (18). This reaction, with an isosbestic point at 393 nm, takes place with a second-order rate constant of 2.0 × 10^10 M^−1 s^−1. In the present studies it was always possible to use sufficiently high enzyme concentrations (100 μM or greater) and low doses (about 20 grays, yielding 14 μM N-methyl nicotinamide radical immediately after the radiolytic pulse) to reduce the extent of the dimerization reaction to zero within experimental error.

Absorption spectra were obtained with a Pye Unicam SP8-200 UV/visible spectrophotometer, and routine enzyme assays with a Pye Unicam SP9506 thermostated spectrophotometer. Enzyme assay following the radiolytic experiments indicated negligible loss of enzyme activity over the course of the experiments described herein, consistent with previous observation (8).

Alloxanthine and N-methyl nicotinamide were purchased from Sigma, United Kingdom. 5-Deazaluminflavin was the generous gift of Dr. Vincent Massey, Dept. of Biological Chemistry, University of Michigan. All other reagents were obtained commercially and used without further purification.

RESULTS AND DISCUSSION

Reaction of N-Methyl nicotinamide Radical with Alloxanthine-blocked Xanthine Oxidase—Previous work indicated that a series of viol met compounds preferentially introduced radiolytically generated reducing equivalents into xanthine oxidase at the flavin center of the enzyme and that subsequent equilibration of the reducing equivalent between the flavin and iron-sulfur centers of the enzyme could be followed spectrophotometrically. No equilibration processes involving the molybdenum center of the enzyme were observed in these experiments, however, due to the low reduction potential of this center relative to the other sites in the enzyme. In order to obtain rates for redox equilibration between the molybdenum center and the other sites of the enzyme, an effort was made to find a compound that introduced reducing equivalents at the molybdenum center instead. N-Methyl nicotinamide (NMN) is known to be reduced by radiolytically generated e\text{aq}− and CO_2, and to have a very low one-electron reduction potential (E^0 = −1.01 V versus SHE; Ref. 19). In contrast to previous work (20), NMN is found to be a very slow substrate for xanthine oxidase, presumably being hydroxylated at the 6-position in a reaction analogous to that catalyzed by aldehyde oxidase (21). Radiolytically generated N-methyl nicotinamide radical (NMN') is thus likely to bind at and reduce the molybdenum center. A potential complication in using NMN' in radiolytic studies is its tendency to dimerize (18), and we therefore investigated the reaction under the conditions appropriate for the enzymatic studies described below. Fig. 1, Panel A, shows the absorption spectra for NMN', NMN, and the dimer species, NMN, obtained by pulse radiolysis of a solution of 2.5 mM NMN' in 20 mM phosphate, pH 6.0. Panel B shows the transient observed at 420 nm, and Panel C a plot of 1/\(A\) versus time for this transient. From the slope of this last plot a second-order rate constant for the dimerization reaction of 2.0 × 10^10 M^−1 s^−1 can be determined under the present experimental conditions. The reaction is sufficiently slow that the experimental conditions (radiolytic dose, enzyme concentration) can be adjusted such that the dimerization reaction is minimized (see below). Even in those cases where dimerization is appreciable, at the 393-nm isosbestic point for the dimerization reaction, all the observed change in absorbance subsequent to the radiolytic generation of NMN' is due to the reaction of the radical with enzyme.

In order to establish whether the radiolytically generated NMN' reacted with an enzyme site other than the molybdenum center, fully functional enzyme was reduced with sodium dithionite, complexed with alloxanthine, and reoxidized. This process yields enzyme having oxidized flavin and iron-sulfur centers, but a redox-inert Mo(IV) center with alloxanthine tightly bound. The pulse radiolysis of disulfide-blocked alloxanthine-complexed xanthine oxidase at pH 6.0 in the presence of 2.5 mM N-methyl nicotinamide, as followed at 525 nm, is shown in Fig. 2. The reaction is found to be markedly biphasic subsequent to the radiolytic generation of NMN'. The wavelength dependence of the kinetics is shown in Fig. 2, Panel A, and is consistent with the rapid reduction of one of the two iron-sulfur centers of xanthine oxidase by NMN' followed by the transfer of the electron equivalent thus introduced into the enzyme from the iron-sulfur center to the flavin, forming the neutral semiquinone. A typical transient obtained at 525 nm is shown in Fig. 2, Panel B, on both short and long time scales. The fast phase is characterized by an absorbance decrease (k_{obs} = 8.6 × 10^10 M^−1 s^−1, Fig. 2C, trace 1), and the slow phase by an absorbance increase (k_{obs} = 125 s^−1, Fig. 2C, trace 2). The slow phase of the reaction decreases on reducing the enzyme from 75 to 27 μM, while the fast phase decreases from 8.6 × 10^10 M^−1 s^−1 to 3.7 × 10^10 M^−1 s^−1, indicating a second-order rate constant for the initial reduction of the enzyme by NMN' in the fast phase of 1.1 × 10^10 M^−1 s^−1. The observed rate
constant of 125 s⁻¹ for the slow phase of the reaction in the present experiment is comparable with the 155 s⁻¹ observed for electron equilibration between the flavin and Fe/S I from pH jump experiments (7), particularly when the difference in temperature in the two studies is taken into account (20 versus 25 °C, respectively).

At pH 6.5, the slow phase in the reaction of NMN⁺ with alloxanthine-complexed xanthine oxidase is lost, consistent with the known pH dependence of the distribution of reducing equivalents within xanthine oxidase, where at high pH iron-sulfur reduction predominates over that of the flavin (21). On the basis of the complete absence of electron transfer from the iron-sulfur center initially reduced by NMN⁺ to the flavin, it is tempting to conclude that it is Fe/S II (E° = -225 mV versus SHE; Ref. 21) that is reduced by NMN⁺ rather than Fe/S I (E° = -330 mV), since a significant amount of electron transfer (at least transiently) from Fe/S I to FAD (E° = -320 mV for the FAD/FADH⁺ couple) would be expected on the basis of their comparable reduction potentials. It is not possible to exclude the possibility, however, that Fe/S I is reduced initially by NMN⁺ followed by electron transfer to Fe/S II, since the latter process would not give rise to a detectable absorbance change (the two iron-sulfur centers have nearly identical absorbance changes upon reduction; Ref. 22). Without knowing which iron-sulfur center is involved in the electron transfer to the flavin, it is impossible to estimate microscopic rate constants for the forward and reverse electron transfer processes between the flavin and iron-sulfur center in the pH 6 experiment. On the basis of the absorbance changes seen at 550 and 470 nm in the fast phase of the reaction (the latter being corrected for the absorbance change due to oxidation of NMN⁺) it is possible to estimate the amount of iron-sulfur versus flavin reduction in the initial enzyme reduction event. The observed ratio of 2.6 is very close to the ratio of 2.8 observed for the iron-sulfur centers of xanthine oxidase (23), and we conclude that NMN⁺ does not react appreciably with the flavin.

**Reaction of NMN⁺ with Desulfo Xanthine Oxidase**—With the reaction of NMN⁺ with the iron-sulfur center(s) of xanthine oxidase characterized, studies were next undertaken with enzyme that was not blocked at the molybdenum center. In order to avoid heterogeneity at the molybdenum center and the slow catalytic reduction of xanthine oxidase by NMN⁺, the desulfo form of the enzyme was used. While this form of the enzyme is catalytically inactive, from the standpoint of electron transfer within the enzyme there is every reason to believe that the inactive dixo molybdenum center behaves analogously to the functional oxo-sulfido molybdenum form (the 80-mV lower reduction potential of the desulfo molybdenum center relative to the functional form at pH 6 being taken into account; Ref. 21).

The reaction of desulfo xanthine oxidase with NMN⁺ at pH 6.0 is superficially quite similar to the reaction with alloxanthine-blocked enzyme, consisting of a biphasic transient subsequent to the radiolytic generation of the radical species (Fig. 3). As with the alloxanthine-blocked enzyme, the slow phase of the reaction has a wavelength dependence consistent with it being due to electron transfer from an iron-sulfur center to the flavin (Fig. 2, Panel A), and is characterized by a rate constant of 125 s⁻¹ (Fig. 3, Panel B) that is independent of enzyme concentration. The slow phase in the reaction of NMN⁺ with desulfo enzyme largely disappears upon increasing the pH to 8.5 (Fig. 3, Panel C), as is the case with the alloxanthine-blocked enzyme.

The fast phase of the reaction of desulfo xanthine oxidase with NMN⁺ is both faster and more complicated than is observed with the alloxanthine-blocked enzyme. The kinetics are most straightforward when the reaction is followed at 598 nm, an isosbestic point for the dimerization of NMN⁺ (24). As shown in Fig. 3, the absorbance change at 598 nm in the fast phase is well described by a single exponential decay, the rate constant of which is independent of enzyme concentration. The rate constant measured at 598 nm (2.8 s⁻¹) is in good agreement with the results obtained using alloxanthine-blocked enzyme. The absorbance change at 598 nm at pH 8.5 is characterized by two exponential decays, the faster component decaying more rapidly as the pH is raised from 6.0 to 8.5. The rate constant of the faster phase decreases from 10 s⁻¹ at pH 6.0 to 0.5 s⁻¹ at pH 8.5. The rate constant of the slower phase is independent of pH (0.5 s⁻¹).

**Fig. 2.** The reaction of radiolytically generated NMN⁺ with alloxanthine-complexed xanthine oxidase at pH 6. A, kinetic intermediates observed upon the pulse radiolysis (22 grays in 100 ns, producing a radical yield of 15 μM) of the complexed enzyme (70 μM) in N₂O-saturated phosphate buffer (20 mM, pH 6.0) containing sodium formate (0.1 mM) and NMN⁺ (2.5 mM). The visible absorption spectrum of NMN⁺ (circles, measured immediately after the pulse) decays rapidly to a different spectrum (squares, measured at 0.5 ms) which in turn converts to a third on a longer time scale (triangles, measured at 25 ms). The last two spectra have been corrected to reflect the entirety of the spectral change for the kinetic phases they represent. B, a typical transient displaying both the fast phase (Trace 1) and the slower phase (Trace 2). C, semilogarithmic plots for the traces displayed in B (one half-life equals 2 divisions) from which the rate constants of 8.6 × 10⁶ s⁻¹ (trace 1) and 125 s⁻¹ (trace 2) are derived. Arrows in Panels B and C indicate the appropriate x axes for each transient.

**Fig. 3.** The reaction of radiolytically generated NMN⁺ with desulfo xanthine oxidase. A, the wavelength dependence of the absorbance change (the fast (circles) and slow (squares) phases of the reaction at pH 6.0 (filled symbols) and 8.5 (open symbols). B, the transient observed at 525 nm upon radiolysis (24 grays in 100 ns) of 100 μM desulfo xanthine oxidase in N₂O-saturated phosphate buffer (20 mM, pH 6.0) containing sodium formate (0.1 mM) and NMN⁺ (2.5 mM). The rate constant obtained from a semilogarithmic plot for the fast phase of the reaction (not shown) is 125 s⁻¹, in good agreement with the results obtained using alloxanthine-blocked enzyme. C, the transient observed upon radiolysis (19 g/100 ml in 100 ns) of 49 mM desulfo enzyme in N₂O-saturated pyrophosphate buffer (20 mM, pH 8.5) containing sodium formate (0.1 mM) and NMN⁺ (2.5 mM).
dase with NMN' and it is evident that unblocking the molybdenum center substantially accelerates the rate of electron entry into the enzyme. An examination of the semilogarithmic plots of the data (Fig. 4, Panel B) demonstrates that whereas the fast phase of the reaction of alloxanthine-blocked enzyme is a well-behaved single exponential ($k_{\text{obs}} = 8.6 \times 10^4 \text{ s}^{-1}$), the reaction with desulfo enzyme is biphasic ($k_1 = 3.6 \times 10^4 \text{ s}^{-1}$ and $k_2 = 8.5 \times 10^3 \text{ s}^{-1}$), with the faster component accounting for the entirety (within experimental error) of the absorbance decrease due to consumption of NMN'. Fig. 5 summarizes the enzyme concentration dependence of the fast phase of the reaction of the desulfo and alloxanthine-complexed forms of xanthine oxidase with NMN' at both pH 6 and 8.5. The second-order rate constant obtained for the reduction of desulfo xanthine oxidase by NMN' is $9.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at both pH 6.0 and 8.5. The rate of reduction of the alloxanthine-blocked enzyme is also pH independent at a rate of $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The slower component of the fast phase is seen to be independent of both enzyme concentration and pH with a rate constant of $8.5 \times 10^3 \text{ s}^{-1}$. On the basis of the substantially faster rate of initial enzyme reduction with desulfo xanthine oxidase compared with the alloxanthine-blocked enzyme, the absence of the absorbance change expected for oxidation of NMN' in the slower kinetic component of the fast phase of the reaction with desulfo enzyme as well as its independence of enzyme concentration, we conclude that NMN' reacts principally at the molybdenum center of the enzyme (first component of the fast phase), followed by internal electron transfer from the molybdenum center (or both) of the iron-sulfur centers (second component of the fast phase).

**Reaction of NMN' with Flavin-alkylated Xanthine Oxidase**—The reaction of flavin-alkylated xanthine oxidase (containing a covalently modified and redox-inert flavin; Ref. 24) in 20 mM phosphate buffer, pH 6.0, followed at 470 and 550 nm, is shown in Fig. 6, Panel A. Comparison with the transient shown in Fig. 2 clearly demonstrates that chemical modification of the flavin results in the loss of the large absorbance increase above 500 nm seen on long time scales in the reaction with enzyme containing an intact flavin, providing independent corroboration that the 125 s$^{-1}$ process observed with desulfo and alloxanthine-blocked enzyme involves electron transfer from an iron-sulfur center to the flavin. With flavin-alkylated enzyme, a much smaller spectral change is seen on the millisecond time scale, which decreases at all wavelengths. This kinetic phase, having a $k_{\text{obs}} = 90 \text{ s}^{-1}$, presumably takes place in the reaction with enzyme containing unblocked flavin, but is obscured by the more extensive spectral change associated with electron transfer from iron-sulfur to flavin. The wavelength dependence of the slow phase of the reaction with flavin-alkylated xanthine oxidase, like that of the more
extensive fast phase, indicates that it is due to iron-sulfur reduction (data not shown).

The observed kinetics can be understood in the context of Scheme 1. It is assumed that electron transfer between each of the three redox-active sites in the (flavin-alkylated) enzyme is freely reversible, and that each kinetic phase represents an approach to an equilibrium distribution

\[ Fe/S I \rightleftharpoons Fe/S II \]

\[ Mo(VI) \rightleftharpoons Fe/S I \]

of reducing equivalents between specific pairs of sites (i.e. \( k_{obs} \) is the sum of the microscopic rate constants for the forward and reverse electron transfer processes). At pH 6.0 the reduction potentials of the Fe/S I, Fe/S II, and the Mo(VI/V) (desulfo) couples are \(-260, -160, \) and \(-320 \) mV, respectively (21). The much more extensive fast phase of the reaction most likely corresponds to electron equilibration between the molybdenum center and Fe/S I, since this center has a reduction potential such that the molybdenum center should be largely (but not completely) oxidized at equilibrium. The slower kinetic phase then corresponds to equilibration between molybdenum and Fe/S II, resulting in the removal of the remainder of the electron density from the molybdenum center. Equilibration between the molybdenum and Fe/S II may take place either directly (in which case equilibration between the two iron-sulfur centers must be slower than 90 s\(^{-1}\)) or mediated via Fe/S I (in which case the 90 s\(^{-1}\) process reflects equilibration between the two iron-sulfur centers). The assignment of Fe/S I as the center equilibrating rapidly with the molybdenum center is consistent with the observation that Fe/S II, but not Fe/S II, exhibits a moderately strong magnetic interaction with the molybdenum center (25) and with the conclusion that the dead time spectral change in pH jump experiments is due to electron equilibration between molybdenum and Fe/S I (7). The possibility that Fe/S II is the site equilibrating rapidly with the molybdenum center cannot be rigorously excluded on the basis of the present kinetic data, however.

At pH 8.5, the slow phase seen with flavin-alkylated enzyme is more prominent (Fig. 6, Panel B), consistent with the lower molybdenum reduction potential relative to those of the iron-sulfur centers at the higher pH (21). The kinetics and wavelength dependence of the slow phase are quite similar to the case with enzyme that has an intact flavin (Fig. 3, Panel C), consistent with the minimal role of the flavin center in electron equilibration at the higher pH. The observation of similar kinetic behavior with desulfo and flavin-alkylated enzyme at pH 8.5 supports the conclusion that the observed slow equilibration between the molybdenum and one of the iron-sulfur centers indeed occurs in enzyme that has an intact flavin, but is obscured by the accumulation of flavin semiquinone when the experiment is performed at low pH.

**Reaction of Radiolytically Generated 5-deazalumiflavin Radical (dF1') with Desulfo Xanthine Oxidase.—** The radical of 5-deazalumiflavin (dF1') has been used as proximal reductant of xanthine oxidase in flash photolysis experiments (5, 6). The pulse radiolytic behavior of 5-deazalumiflavin has also been investigated (26, 27). In light of the appearance of relatively large spectral change observed in the transients at long time (\( k_{obs} \sim 12 \) s\(^{-1}\)) that have recently been attributed to an unspecified artifact in the flash photolysis experiments (6), the reaction of radiolytically generated dF1' with desulfo xanthine oxidase was examined. A transient of the reaction at pH 6.1, as observed at 525 nm, is shown in Fig. 7, Panel B.

The transient is distinctly triphasic, being characterized by a very rapid increase in absorbance on a <10-µs time scale, followed by an absorbance decrease on a time scale of 100's of microseconds, and finally an absorbance increase on a millisecond time scale. Significantly, no very slow kinetic phases (\( k_{obs} \sim 10 \) s\(^{-1}\)) are observed. As with NMN', dF1' dimerizes and complicates the kinetics at short time (\( k_{dimerization} = 7.0 \times 10^8 \) M\(^{-1}\) s\(^{-1}\); Ref. 26). The effect on the kinetics is minimal, however, given the lower rate of dimerization compared to NMN', and the fact that the dF1 dimer does not absorb significantly above 350 nm (27). Because of this and limitations in the amount of deazaflavin available for these studies, analysis of the fast phase was not extended beyond noting that \( k_{obs} \) for the fast phase of 2.2 \( \times 10^4 \) s\(^{-1}\) at an enzyme concentration of 88 nM yields a second-order rate constant for the reduction of desulfo xanthine oxidase by dF1' of 2.5 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\), in good agreement with the value of 2.0 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\) determined at pH 7.2 by flash photolysis (6), but somewhat slower than the 9.0 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\) seen with NMN'. A semilogarithmic plot of the slow phase of the transient is shown in Fig. 7, Panel B, and from the slope the rate constant of 145 s\(^{-1}\) for the slow phase is obtained, in good agreement with both the results using NMN' and previous pH jump experiments (7). The wavelength dependence of the three phases is shown in Fig. 7, Panel A. These data demonstrate that the first phase involves the formation of the 5-deazalumiflavin radical, the second phase the loss of dF1' and concomitant reduction of both iron-sulfur and flavin (to the neutral flavin semiquinone) on the basis of the relative absorbance changes at 470 and 550 nm, and the last phase electron transfer from iron-sulfur to flavin in that portion of the enzyme which had been reduced by dF1' at the iron-sulfur center. At final equilibrium there is clearly a preponderance of flavin semiquinone formation over iron-sulfur reduction in that portion of the total enzyme population (~10%) that has become reduced by one electron in the radiolytic experiment. This is consistent with the
results of both reductive titrations of enzyme with sodium dithionite (28) and kinetic experiments (9) in which it is demonstrated that at low levels of enzyme reduction at pH 6.0 partially reduced enzyme exhibits greater absorbance at long wavelengths than does oxidized enzyme, due to substantial accumulation of the neutral flavin semiquinone. These observations, and our own presented here, suggest that the reduction potential for the FAD/FADH\(^\text{+}\) couple is comparable to the reduction potential for Fe/S II at pH 6.

It is not possible on the basis of these results alone, however, to establish which (or both) of the iron-sulfur centers of the enzyme is involved in the initial reduction of enzyme by dF1'. Since the final equilibrium in the one-electron reduced enzyme at pH 6 must be between Fe/S II and FAD (on the basis of their high reduction potentials relative to the other two sites), two alternative electron equilibration schemes can be considered. If Fe/S II is directly reduced by dF1', equilibration with the flavin is most likely to proceed directly (since the equilibrium distribution of reducing equivalents between the two iron-sulfur centers substantially favors Fe/S II reduction) and the slow phase thus reflects equilibration between Fe/S II and the flavin. If it is Fe/S I that is initially reduced by dF1', the slow phase must be either the simultaneous equilibration with Fe/S II and the flavin, or rapid equilibration with Fe/S II alone, which subsequently equilibrates with the flavin. While it is unlikely that dF1' reacts exclusively with only one or the other iron-sulfur center, some indication of which is the principal site of reduction by dF1' can be obtained from a comparison of the reaction of the radical with oxidized and partially reduced enzyme at pH 6. Fig. 8 shows the transients observed with oxidized enzyme and enzyme containing on average approximately two electron equivalents per enzyme. At this level of reduction, the distribution of electron equivalents is expected on the basis of the relative reduction potentials (21) to be such that the principal reduced species are Fe/S II and FADH\(^\text{+}\) (with some FADH\(^2\) and Fe/S II now oxidized). If dF1' reduced Fe/S II directly, both the extent and rate of the reaction should decrease significantly relative to oxidized enzyme. A comparison of the transients shown in Fig. 7 indicates that this is clearly not the case, and we infer that it is Fe/S I rather than Fe/S II that is the principal site reduced initially by dF1' with fully oxidized enzyme. It is not possible to distinguish between the alternatives of direct electron transfer between Fe/S I and FAD, and indirect equilibration via Fe/S II given the overall complexity of the observed transients. If the slow phase in the reaction of NMN' with flavin-blocked enzyme is ascribed to equilibration between the two iron-sulfur centers (k\(_{\text{obs}}\) = 90 \(s^{-1}\)), however, electron transfer between the two iron-sulfur centers would be taking place too slowly to mediate equilibration between Fe/S I and the flavin in the reaction of unblocked enzyme with dF1'. As was true in the case of the reaction of desulfo xanthine oxidase with NMN' alkylation of the flavin results in loss of the absorbance increase above 500 nm on the millisecond time scale in the reaction with dF1', demonstrating that the slow phase involves reduction of the flavin site (data not shown). By contrast with the NMN' experiment, however, little if any absorbance change is observed on the millisecond time scale in the reaction with dF1', consistent with preferential reduction of the molybdenum center by NMN' and reduction of the iron-sulfur and flavin centers by dF1'.

The reaction of dF1' with oxidized desulfo xanthine oxidase at pH 8.5 is also triphasic. At 550 nm, however, the slowest phase is manifested as an absorbance decrease rather than the increase in absorbance observed at pH 6. The wavelength dependence of the three phases of the reaction (Fig. 9, Panel A) is consistent with formation of dF1' (fast phase), followed by reduction of the flavin (and iron-sulfur as well, as indicated by the ratio of the absorption changes at 470 and 575 nm for the intermediate phase) and finally transfer of an electron equivalent from the flavin semiquinone to an iron-sulfur center (slow phase, k\(_{\text{obs}}\) = 218 \(s^{-1}\)). The acceptor iron-sulfur center must ultimately be Fe/S II, by virtue of its high reduction potential, although as discussed above the possibility of indirect equilibration via Fe/S I cannot be discounted. The observed rate constant for the slow phase is comparable to that obtained by the pH jump technique for the equilibration of an electron equivalent between Fe/S I and the flavin.

**Fig. 8.** The dependence of the reaction of dF1' with desulfo xanthine oxidase upon the level of enzyme reduction. Experimental conditions were the same as for Fig. 7. A, observed transients at 550 and 470 nm following pulse radiolysis of the oxidized enzyme. B, observed transients at the same wavelengths after the enzyme had been reduced in situ with a train of 10 27-gray pulses, sufficient to reduce the enzyme by two equivalents. Arrows indicate the appropriate y axes for each transient.

**Fig. 9.** The reaction of radiolytically generated dF1' with desulfo xanthine oxidase at pH 8.5. A, spectra formed upon the pulse radiolysis (34 gray in 500 ns) producing a radical yield of 23 \(\mu\)M of the enzyme (156 \(\mu\)M) in \(\text{N}_2\text{O}\)-saturated pyrophosphate buffer (20 mM, pH 8.5) containing sodium formate (0.1 M) and 5-deazali-flavin (100 \(\mu\)M). The visible absorption spectrum of dF1' (circles, measured immediately after the pulse) decays rapidly to a different spectrum (squares, measured at 0.5 ms) which in turn converts to a third on a longer time scale (triangles, measured at 25 ms). The last two spectra have been corrected to reflect the entirety of the spectral change for the kinetic phases they represent. B, a typical transient, observed at 550 nm; C, semilogarithmic plot of the slow phase of the transient displayed in B (one half-life equals 2 divisions) from which the rate constant of 218 \(s^{-1}\) is derived.
CONCLUSIONS

The data presented here provide new insight into electron transfer within xanthine oxidase. A comparison of the results with alloxanthine-blocked and desulfo xanthine oxidase indicates that the molybdenum center is the preferred site of entry of electron equivalents into the enzyme when NMN' is used as reductant \((k_{eq} = 1.1 \times 10^8 \text{ M}^{-1} \text{s}^{-1})\) and \(9.0 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\), respectively), consistent with the known hydroxylation of NMN' by xanthine oxidase. At pH 6.0, reduction of the molybdenum center is followed first by the extremely rapid equilibration \((k_{abs} = 8.5 \times 10^9 \text{ s}^{-1})\) with at least one of the iron-sulfur centers (tentatively assigned Fe/S I), then subsequent equilibration between the iron-sulfur center(s) and the flavin \((k_{abs} = 125 \text{ s}^{-1})\). These assignments are based on the wavelength dependence of the kinetic phases observed in the reaction of NMN' with desulfo xanthine oxidase containing either blocked or unblocked FAD. The observation of transient iron-sulfur reduction in the transfer of electron equivalents from the molybdenum center to the flavin in the reaction of NMN' with desulfo xanthine oxidase unequivocally documents the role of the iron-sulfur centers in mediating electron transfer between the molybdenum center and flavin of xanthine oxidase.

The slow phase in the reaction of NMN' with enzyme containing unblocked flavin is characterized by a large absorbance increase above 525 nm, is unaffected by complexing the molybdenum center with alloxanthine (rendering it redox-inert) but is lost upon covalent modification of the flavin with iodoacetamide. With flavin-alkylated enzyme, a much smaller spectral change is observed on the millisecond time scale \((k_{abs} = 90 \text{ s}^{-1})\) that has the characteristic wavelength dependence of iron-sulfur reduction. This kinetic process is presumably taking place in enzyme having an intact flavin, but is undetected because of the much larger spectral change associated with flavin reduction that occurs at a comparable rate. While it is not possible from the present studies to definitively assign the iron-sulfur center which equilibrates rapidly with the molybdenum center, it appears most likely to be Fe/S I, given the magnetic interaction known to exist between this center and the molybdenum (25). If this assignment is correct, then the slow phase \((k_{abs} = 90 \text{ s}^{-1})\) in the reaction of NMN' with the flavin-blocked enzyme must be due to the equilibration of reducing equivalents between Fe/S I and the molybdenum center. This equilibration may be direct (in which case the observed rate constant is for the equilibration between the molybdenum center and Fe/S I) or mediated by Fe/S I (in which case the rate constant is for the equilibration between the two iron-sulfur centers), as discussed above. Since the equilibrium between the two iron-sulfur centers is not expected to have an absorbance change directly attributed to it, the effect of overall equilibration with the higher potential Fe/S II would be to draw reducing equivalents out of the molybdenum center to a greater extent, with concomitantly greater iron-sulfur reduction. At pH 8.5, the slow phase associated with (further) iron-sulfur reduction predominates on the millisecond time scale since at this pH flavin reduction to the semiquinone is thermodynamically unfavorable.

By contrast with NMN', reduction of desulfo xanthine oxidase with radiolytically generated dF' results predominantly in the initial reduction of flavin and iron-sulfur centers. At both pH 6 and 8.5, the initial reduction of enzyme is followed by a slow spectral change on a millisecond time scale (in favor of further flavin reduction at pH 6 and Fe/S reduction at pH 8.5). The rate constant associated with this slow spectral change at pH 6.0 \((145 \text{ s}^{-1})\) is in good agreement both with the results of the experiments with NMN' as reductant and with previous studies of electron transfer in xanthine oxidase by pH jump. In none of the present studies with radiolytically generated dF' are kinetic processes observed at long time (>10 ms) such as have been reported in studies with photolytically generated radical (5, 6).

The present experimental results are summarized in Scheme II. As discussed above, on the basis of the results using NMN' as reductant of the flavin-blocked enzyme, it is likely that the equilibration between Fe/S I and the molybdenum center is responsible for the faster of the two kinetic phases observed. If this is true the slow phase of the reaction must be either the direct equilibration between the molybdenum center and Fe/S II or the equilibration between the two iron-sulfur centers, with the molybdenum center equilibrating with Fe/S II only indirectly via Fe/S I. Equilibration between the molybdenum center and Fe/S II does not account for more than 20% of the overall oxidation of the molybdenum center, and is slow \((90 \text{ s}^{-1})\) regardless of whether it is direct or mediated by Fe/S I. The fact that the slow process observed in the reaction of NMN' with flavin-alkylated enzyme is independent of pH suggests (but does not prove) that it is due to the equilibration of reducing equivalents between the two iron-sulfur centers. The equilibration of iron-sulfur centers with the flavin observed to take place at 125 s' and 218 s' at pH 6 and 8.5, respectively. These rate constants are consistent with previous work using pH jump, to monitor equilibration between the flavin and Fe/S I (7), and pulse radiolysis studies, using viologen radicals as enzyme reductant to monitor equilibration between flavin and Fe/S II (8). We infer that the two iron-sulfur centers equilibrate with the flavin at comparable rates, which are both moderately dependent upon pH (approximately doubling over the pH range 6 to 8.5). This conclusion is also consistent with recent pH jump experiments utilizing xanthine oxidase containing 8-chloroFAD in place of the native cofactor in which the results have been interpreted as reflecting electron equilibration between flavin and Fe/S II at least as fast as with Fe/S I (29). The value of 125 s' \((\text{pH 6.0, 20 °C})\) for equilibration between the flavin and Fe/S II determined here is comparable to the revised value of 118 s' \((\text{pH 7.2, 25 °C})\) determined by flash photolysis (6). At pH 8.5, however, the discrepancy between the pulse radiolysis and flash photolysis is greater than a factor of 2 \((218 \text{ s}^{-1} \text{ and 100 s}^{-1})\), respectively, neglecting the 5 °C higher temperature at which the latter figure was obtained. Given the general agreement of the pulse radiolysis and pH jump results, and the difficulties in the analysis of the flash photolysis owing to the slow kinetic artifact occurring at ~10 s', we regard the pulse radiolysis values to be more reliable.

The central result of the work described here is that under those conditions where the molybdenum center is the site of electron entry into xanthine oxidase (i.e., using NMN' as reductant), transient reduction of at least one of the iron-sulfur centers is observed in the course of electron migration to the flavin. This observation demonstrates the long hypothesized role of the iron-sulfur centers of xanthine oxidase in
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mediating electron transfer between the molybdenum and flavin centers, the sites of catalytic entry and egress, respectively, of reducing equivalents.

REFERENCES


