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Laser Flash Photolysis Studies of Intramolecular Electron Transfer in Milk Xanthine Oxidase[†]

Anjan Bhattacharyya, Gordon Tollin,* Michael Davis, and Dale E. Edmondson

ABSTRACT: The laser flash photolysis technique has been used to study the reaction of the 5-deazaflavin radical with the redox centers of milk xanthine oxidase under anaerobic conditions. Kinetic studies show that the deazaflavin radical reacts primarily with the functional Mo center, the FAD moiety, and, to a small extent, the Fe/S_I center. The reaction of the photogenerated reductant with the functional Mo center is second order ($k = 6.6 \times 10^7$ M^{-1} s^{-1}), whereas its reaction with the FAD moiety is more rapid ($t_{1/2} < 25$ μs), apparently occurring via a preexisting complex. No evidence was found for the Mo reaction of the 5-deazaflavin radical with the desulfo-Mo center or when the Mo was trapped as Mo^{IV} by complexation with alloxanthine. Kinetic traces observed at 483 nm show the reduction of the two Fe/S centers to be bisphasic, with Fe/S_I being reduced more rapidly ($k = 77$ s^{-1}) than Fe/S_{II} ($k = 12$ s^{-1}). No evidence was found for any direct electron transfer between the two Fe/S centers. Flash photolysis studies with deflavo-xanthine oxidase show that in contrast to other forms of the enzyme which contain FAD, Fe/S_{II} can react directly with the 5-deazaflavin radical in a second-order manner. When a functional Mo center is present, Fe/S reduction occurs either via direct reaction with the 5-deazaflavin radical or via reaction with Mo^V. In the desulfo-deflavoenzyme, reduction of the two Fe/S centers occurs only by direct reaction with the 5-deazaflavin radical. From an analysis of the kinetic data and the reported oxidation–reduction potentials of the centers in xanthine oxidase, the forward and reverse rate constants for each of the inter- and intramolecular electron-transfer processes have been calculated. The results obtained on the one-electron reduced form of the enzyme are inconsistent with the views that (1) equilibration of reducing equivalents among the redox centers in xanthine oxidase occurs much more rapidly than turnover and (2) the distribution observed among the various centers during rapid mixing experiments is dependent on their relative oxidation–reduction potentials.

Mechanistic studies of inter- and intramolecular electron-transfer reactions in enzymes containing multiple redox centers have been and continue to be an area of active research interest (Palmer & Olson, 1980). Due to its ready availability and stability, milk xanthine oxidase has been the subject of numerous kinetic and structural studies [cf. Bray (1975, 1980)]. The enzyme contains one molybdenum, one FAD, and two 2Fe–2S centers in each of its two independent catalytic centers. The Mo center is now generally accepted to be the site of entry of reducing equivalents from the substrate, while the FAD moiety is the site of reduction of O_2 via either a two-electron or a one-electron mechanism (Hille & Massey, 1981; Porras et al., 1981). Results from studies on the deflavo

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enzyme (Komai et al., 1969) have shown that the Mo center is capable of electron donation directly to the 2Fe–2S centers, as well as to the FAD. Olson et al. (1974) were the first to propose that internal electron transfer among the various redox centers was more rapid than catalytic turnover. They cited as support for this hypothesis the pH-jump rapid-freeze electron-spin resonance (ESR) experiments of Edmondson et al. (1973) as well as the success of calculations based on the relative oxidation–reduction potentials of the redox centers in describing stopped-flow, rapid-freeze quench, and steady-state kinetic data. Recent potentiometric titrations of the enzyme at different pH values (Porras & Palmer, 1982; Spence et al., 1982) have established the respective redox potentials for each redox center.

The rapid equlibrium hypothesis is quite attractive in describing the known kinetic properties of xanthine oxidase, and thus it is desirable to obtain a direct measurement of electron-transfer rates among the individual redox centers of the enzyme. These reactions are thought to be quite rapid ($k > 10^2\ s^{-1}$) and have not been resolvable by normal rapid mixing procedures (Palmer & Olson, 1980). Temperature-jump kinetic studies on partially reduced samples of enzyme have also been reported to be unsuccessful (Fischer & Hurst, 1978), presumably as a consequence either of a temperature insensitivity of the various redox equilibria or of an identical enthalpy change for each of the equilibria involved. Thus, in order to reduce the enzyme rapidly by using nonmixing techniques, we have employed laser flash photolysis. Previous work in our laboratory (Cusanovich & Tollin, 1980; Ahmad et al., 1982; Tollin et al., 1982) has demonstrated the utility of this technique in the study of intramolecular electron transfer in several redox enzyme systems. Massey & Hemmerich (1978) have shown the feasibility of using 5-deazaflavin in the photochemical reduction of low-potential redox groups in biological systems. This facile reactivity is due to the very low potential (−0.65 V at pH 7.0) of the 5-deazaflavin radical (Blankenhorn, 1976). Since the redox centers in xanthine oxidase at pH 7.0 are in the potential range of −0.35 to −0.2 V, it appears reasonable to expect that the deazaflavin radical rapidly generated by the laser flash would be an effective reductant of the enzyme. Furthermore, the experimental conditions can be adjusted such that the concentration of 5-deazaflavin radical generated in a single flash is sufficiently lower than that of xanthine oxidase to ensure that no single molecule of enzyme is reduced by more than one electron. The rate of distribution of that single reducing equivalent among the various redox centers can then be followed spectrally on a time scale ranging from microseconds to milliseconds. This paper describes the results of such experiments on various forms of xanthine oxidase in which the kinetic properties of enzyme preparations modified at the molybdenum and flavin centers have been investigated and compared with results obtained with the native enzyme.

Materials and Methods

Xanthine oxidase was isolated from fresh raw buttermilk according to the procedure of Massey et al. (1969). The purified enzyme preparations exhibited A_{280}:A_{450} spectral ratios between 5.2 and 5.5 and exhibited activity:flavin ratio (AFR) values consistent with 50–60% functionality. Analyses of two representative enzyme preparations gave Mo:FAD ratios of 0.9–1.0. The deflavoenzyme was prepared either as described by Komai et al. (1969) or by the KI procedure outlined by Kanda et al. (1972). No spectral or kinetic differences were observed to distinguish the two types of preparations. Desulfo-xanthine oxidase was prepared as described by Massey & Edmondson (1970). Alloxanthine-complexed enzyme was prepared by reduction of the enzyme with dithionite in the presence of 1 mM alloxanthine. Removal of excess reagents and reoxidation of the FAD and Fe/S centers were achieved by passing the sample down a G-25 (1 × 10 cm) column equilibrated with 20 mM phosphate–0.3 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0. Preparations of xanthine oxidase in which the FAD moiety is alkylated by iodoacetamide were prepared by using dithionite-reduced enzyme as described by Komai & Massey (1971). 5-Deazariboflavin was a gift from Dr. C. Cheng, Midwest Research Institute, Kansas City, MO. All other chemicals used were of reagent grade from commerically available sources. Although most of the experiments were performed at pH 7.0, some measurements were also made at pH 8.5.

Deoxygenation was carried out in a 0.8-cm glass cell fitted with a vacuum stopcock. Initially the sample was brought to ice temperature, and an indirect vacuum was applied to prevent foaming and denaturation of the protein sample. Once all bubbles were removed from the sample, the cell was brought to room temperature by immersion in a water bath followed by another evacuation. This cycle was repeated about 4 times. The transient signals themselves, i.e., FAD reduction, Fe/S center reduction, and deazaflavin radical formation and decay (see below), provided a good indicator of the degree of oxygen removal. Once this was accomplished, the kinetics were quite reproducible from sample to sample.

The flash photolysis apparatus utilized a pulsed nitrogen laser (PRA LN100) to pump a dye laser consisting of a 1.2 mM solution of 1,4-bis(2-methylstyryl)benzene (Bis-MSB) in *p*-dioxane in a 1-cm path-length quartz rectangular cuvette. The stimulated emission provided a pulse of light at 420 nm with a duration of approximately 300 ps. This served as the source of excitation for the deazaflavin which, via reduction of its triplet state by EDTA, generates deazaflavin semiquinone. Although xanthine oxidase also absorbs at this wavelength, control experiments demonstrated that no reduction occurred in the absence of the deazaflavin. However, this absorption limited the range of enzyme concentrations over which experiments could be effectively performed. Optical transients were monitored by focusing monochromatic visible light through the sample onto an RCA 4463 photomultiplier tube (S20 response). The laser excitation beam illuminated the sample at an angle of about 10° to the monitoring beam. A Nicolet 1170 signal averager accumulated the optical transients which were then fed into a strip chart recorder. Normally, 1000 data points were obtained along the decay curve, and 16–32 flashes were signal averaged per kinetic trace. All kinetic data were obtained at ambient temperatures (23–25 °C).

Results

Kinetic Studies of the Native Enzyme. The visible spectrum of xanthine oxidase in its oxidized form is comprised of contributions from the FAD moiety (λ_{max} 450 nm) and from the two 2Fe–2S centers which have broad, featureless transitions in the visible region. Spectral studies of the enzyme during stepwise reduction by dithionite (Porras & Palmer, 1982; Olson et al., 1974) indicate that the two 2Fe–2s centers do not contribute equally to the decrease in absorbance observed on reduction of the enzyme. A possibility has been suggested (Porras & Palmer, 1982) that the molybdenum center also contributes to the visible absorption spectrum; to date, there is no direct evidence for this hypothesis.

Figure 1 shows flash-induced difference spectra for xanthine oxidase measured at 50 and 400 ms after the laser flash. These

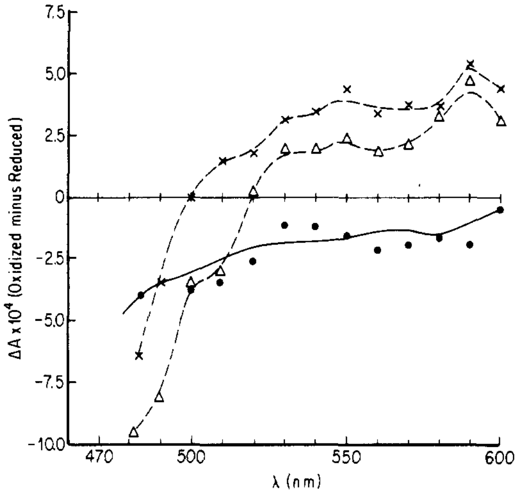

FIGURE 1: Flash-induced difference spectra (oxidized minus reduced) observed at 50 and 400 ms after the laser flash for 20 μM xanthine oxidase in the presence of 70 μM 5-deazariboflavin. The buffer used was 20 mM potassium phosphate–20 mM EDTA, pH 7.0: (×) 50 ms; (△) 400 ms; (●) 50 ms, 50% reduced prior to flash; (—) 50% oxidized minus fully reduced (data normalized at 483 nm to flash data).

are consistent with the formation and partial decay of the FAD radical (wavelength region 510–600 nm) and with the formation of reduced Fe/S centers (wavelength region 480–510 nm).[1] The relative signal intensities at 483 and 580 nm are approximately 2.9. This is in agreement with reasonable values for the relative difference extinction coefficients of oxidized–reduced Fe/S centers and FAD/FADH·. Three kinetically separable optical transients are observed at 483 nm following the laser-induced formation of the deazaflavin radical (Figure 2). The absorptions of the oxidized and neutral semiquinone forms of the flavin are isosbestic at this wavelength,[2] and thus the reduction of the FAD center will not contribute. Initially a rapid increase in absorption due to the formation of the deazaflavin radical (Edmondson et al., 1972a) is observed which subsequently decays in a first-order manner (Figure 2c)(k = 530 s^{-1}) (see below) due to its oxidation by the redox centers in the enzyme. The corresponding rapid absorption increase and slower decay observed with deazaflavin in the absence of xanthine oxidase is shown for comparison in Figure 2d. Subsequent to the oxidation of the deazaflavin radical, bleaching of the Fe/S centers is observed at a rapid (k = 77 s^{-1}, Figure 2b) and a slow (k = 12 s^{-1}, Figure 2a) rate. Both of the observed rates of Fe/S reduction follow first-order kinetic behavior (within experimental uncertainity). Analysis of the kinetic data from several batches of enzyme shows that the relative amplitude of the rapid and slow phases of Fe/S reduction is somewhat variable, ranging between 1:1 and 1:2 depending upon the sample.

The kinetic properties of enzyme-bound FAD radical formation and decay were monitored at 590 nm (Figure 3). Changes in the absorption of the Fe/S centers are small at this wavelength compared to the absorption of the neutral FAD radical (pK_a = 9.5; Porras & Palmer, 1982). The data in Figure 3 show that the rate of FAD radical formation is very fast (half-time ≤25 μs). This is within the time resolution of the apparatus under present conditions, which is estimated to be 10–50 μs with these enzyme samples due to scattering artifacts. If this were a second-order reaction, its rate constant would be ≥1.4 × 10^9 M^{-1} s^{-1}, a rate which is close to being diffusion controlled. However, as we will discuss below, this reaction probably proceeds via a preexisting complex. Only a small amount of decay of the FAD radical is observed at times up to 400 ms. Thus, either this species does not appreciably reduce any of the other redox centers in the enzyme

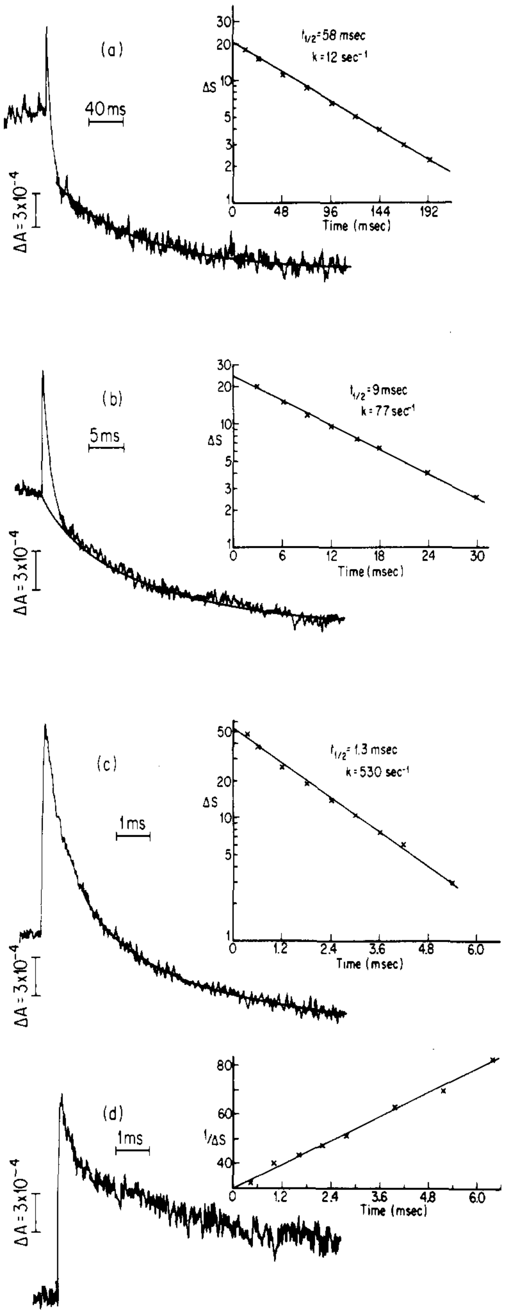

FIGURE 2: Transient absorbance changes observed at 483 nm upon laser flash photolysis of 20 μM xanthine oxidase in the presence of 70 μM 5-deazariboflavin under anaerobic conditions. (a) 400-ms time scale; (b) 50-ms time scale; (c) 10-ms time scale; (d) deazariboflavin alone, 10-ms time scale. The enzyme was 50% functional, and the buffer used was 20 mM potassium phosphate–20 mM EDTA, pH 7.0. Inserts in (a), (b), and (c) represent plots of exponential fits to the decay curves in these time ranges [shown in the traces as superimposed solid lines; in (c), the theoretical line is not extended back to zero time for purposes of clarity]. The insert in (d) is a second-order plot for deazaflavin radical disproportionation. The apparent first-order kinetic constants included with each trace were obtained from the slopes of these plots.

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[1] It is technically difficult to make observations much below 480 nm due to reduction of the sample by the measuring beam.

[2] This is only an apparent isosbestic point determined by using the present optical system. It was empirically determined as the wavelength at which rapid FAD reduction was not observed, i.e., at which traces such as that shown in Figure 2b extrapolated to the original base line at zero time.

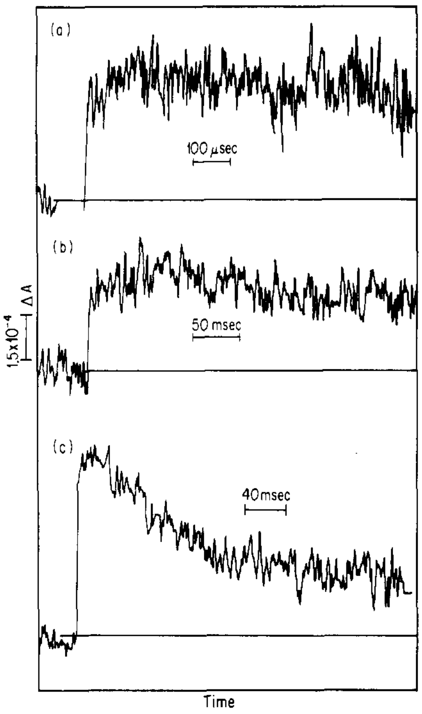

FIGURE 3: Transient absorbance changes observed at 590 nm upon laser flash photolysis of 20 μM xanthine oxidase in the presence of 70 μM 5-deazariboflavin (a, b). The experimental conditions are identical with those in Figure 2 except that the sensitivity is twice as great. Part c is the transient obtained with arsenite-treated xanthine oxidase.

or reduction of FAD to the FAD radical by the reduced molybdenum is occurring within the sample at a rate comparble to the oxidation of the FAD radical by an Fe/S center, thus leading to only a small net change in the 590-nm absorbance. We will show below that the latter is probably the case, inasmuch as when the molybdenum is nonfunctional, appreciable FAD radical decay is observed. Furthermore, at pH 8.5, where the potential difference between FAD and Fe/S is greater than at pH 7.0, we also observe FAD radical decay.

Effect of Enzyme Concentration on the Observed Kinetic Properties. In order to determine which optical transients are due to intramolecular electron transfer and which are due to the direct reduction by the photogenerated deazaflavin radical, the concentration of the enzyme was varied while the deazaflavin concentration was kept constant. Any observed concentration dependence would reflect a second-order reaction with the deazaflavin radical. The only observed spectral transient exhibiting such behavior was the decay of the deazaflavin radical itself (Figure 4), with a calculated second-order rate constant of 3.3×10^7 M^{-1} s^{-1}. This value, however, must be modified to correct for the concentration of nonfunctional enzyme in the sample (~50%). As we will show below, the desulfo-enzyme does not react with the deazaflavin radical in a second-order manner. Thus, the corrected second-order rate constant is 6.6×10^7 M^{-1} s^{-1}. No dependence on concentration was found for any of the other transients observed at 483 nm. The rate of FAD radical formation was too rapid to follow (see above), and thus its concentration dependence could not be determined. The very rapid rate observed for its formation ($t_{1/2} \leq 25$ μs) is faster than the rate of the majority of the 5-deazaflavin semiquinone oxidation ($t_{1/2} = 2$ ms). These data suggest that the deazaflavin radical reacts directly with the FAD moiety and with the molybdenum center (which is not expected to give any color change on reduction). This will be considered further below.

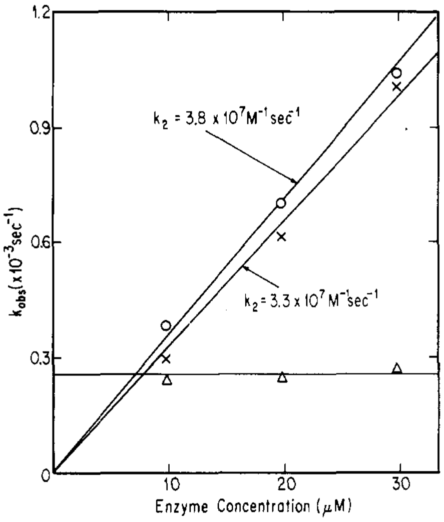

FIGURE 4: (×) Dependence of k_{obsd} for deazariboflavin radical decay upon xanthine oxidase concentration. (△) Dependence of k_{obsd} for deazariboflavin radical decay upon alloxanthine-treated xanthine oxidase concentration. (○) Dependence of k_{obsd} for deazariboflavin radical decay upon deflavo-xanthine oxidase concentration. Conditions as in Figure 2.

If it is assumed that FAD radical formation is a second-order process ($k \geq 1.4 \times 10^9$ M^{-1} s^{-1}), then it must proceed at a rate which is more than an order of magnitude greater than the rate of deazaflavin radical reaction with molybdenum ($k = 6.6 \times 10^7$ M^{-1} s^{-1}). Thus, it would be expected that the FAD center would be the predominant site for electron entry into the enzyme. This is not supported by the experimental data since large quantities of deazaflavin radical are oxidized via the slower rate process. The only interpretation which would be consistent with these data is to postulate that an oxidized deazaflavin–enzyme–FAD complex exists prior to electron transfer and that reduction of FAD occurs via a first-order intramolecular process subsequent to photochemical reduction of the protein-bound deazaflavin ($k \geq 2.8 \times 10^4$ s^{-1}). An analogous complex was suggested for the reduction of *Chlorobium* cytochrome c_{552} by photoreduced lumiflavin (Tollin et al., 1982). Difference spectral and gel filtration studies showed no observable binding of oxidized deazaflavin to the oxidized enzyme under the conditions (pH, buffer, reactant concentration) of the flash experiments. However, the amount of xanthine oxidase reduced per flash is very small (~3×10^{-8} M), and thus the detection of such a small amount of complex would be exceedingly difficult using these techniques. Indeed, under the experimental conditions used, a dissociation constant of 50 mM can be calculated for this complex if a quantum yield for photoreduction of 100% is assumed. Furthermore, as we shall show below, the existence of a complex is consistent with the results obtained with modified xanthine oxidases, where fast FAD reduction occurs under conditions in which deazaflavin radical decay is observed to be independent of enzyme concentration.

Kinetic Properties of Partially Reduced Enzyme. In order to distinguish which Fe/S center is rapidly and which is slowly reduced upon laser photolysis, flash experiments were performed on enzyme samples at various stages of reduction (accomplished by steady-state illumination). The rationale for this approach is that due to the difference in the oxidation–reduction potentials of the two Fe/S centers (0.1 V; Porras & Palmer, 1982), it should be possible to observe the kinetic properties of partially reduced samples of enzyme in which Fe/S_{II} is substantially reduced while Fe/S_I is substantially oxidized. If, for example, the rapid Fe/S reduction

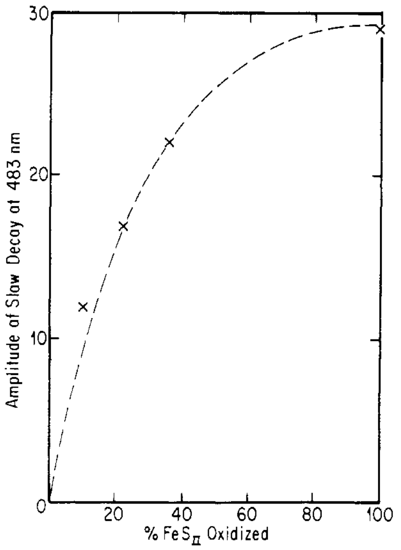

FIGURE 5: Dependence of the amplitude of slow Fe/S reduction after laser excitation upon the extent of prior reduction of xanthine oxidase. Conditions as in Figure 2. The dashed line is the exponential fit to the data.

is due to Fe/S$_I$ and the slow rate is due to Fe/S$_{II}$, one should observe decreasing amounts of the slow component of Fe/S reduction as the enzyme is progressively reduced. The fractional degree of Fe/S$_{II}$ reduction (as well as the other redox centers in the enzyme) was estimated by using the redox potential data of Porras & Palmer (1982) and the computational method described by Olson et al. (1974) and by Hille et al. (1981).

Figure 1 shows a flash-induced difference spectrum obtained 50 ms after the flash with an enzyme sample which has been approximately 50% reduced. It is apparent that the positive absorbance change due to FAD radical formation has disappeared (consistent with the redox potentials which predict that 80% of the FAD would be reduced under these conditions) and that Fe/S reduction is diminished. Also shown for comparison is the 50% oxidized minus reduced spectrum obtained in a spectrophotometer after complete photochemical reduction of this sample. As can be seen, the agreement is satisfactory. From redox potential considerations (Porras & Palmer, 1982), this should correspond to the difference spectrum for reduction of Fe/S$_I$. This supports the assignment that the slow phase of reduction is in fact due to Fe/S$_{II}$. The data in Figure 5 show that upon reduction of the enzyme the slow Fe/S decay decreases in amplitude in proportion to that calculated for the reduction of Fe/S$_{II}$. The decay rate remains relatively constant. Over this same level of reduction, only 15% of Fe/S$_I$ is calculated to be reduced, and correspondingly, we find little or no change in the amplitude of the fast Fe/S decay. These kinetic data provide additional support for the suggestion that the slow Fe/S reduction rate is due to the high-potential Fe/S$_{II}$ and that the rapid rate is due to the reduction of the lower potential Fe/S$_I$. We also find that the rate of deazaflavin radical decay is unchanged upon partial reduction, which is consistent with the molybdenum–deazaflavin radical reaction proposed above. We conclude that Fe/S$_I$ reduction proceeds via electron transfer from Mo^V formed as a result of the reaction of Mo^{VI} with deazaflavin radical.

Kinetic traces obtained from flash experiments carried out at pH 8.5 indicate that the reduction of both Fe/S centers and the oxidation of the deazaflavin radical occur at rates comparable to those observed at pH 7.0 (data not shown). The only difference in kinetic behavior between pH 8.5 and pH 7.0 is observed at 590 nm where virtually complete decay of the protein-bound FAD radical is found to occur at a rate identical with that of reduction of Fe/S$_{II}$. This difference in behavior may be explained on the basis of the changes in midpoint potentials of the FAD/FADH· and Fe/S$_{II(ox)}$/Fe/S$_{II(red)}$ couples. The redox potential difference is larger at pH 8.5 (−90 mV) than at pH 7.0 (−38 mV). Hence, from a thermodynamic point of view, FADH· functions as a stronger reductant for oxidized Fe/S$_{II}$ at this pH. These experiments are thus consistent with electron flow from reduced FAD to oxidized Fe/S$_{II}$ (see below).

Kinetic Studies of the Desulfo-Enzyme. It is well-known that all preparations of xanthine oxidase contain varying amounts of nonfunctional enzyme. This nonfunctionality results from loss of a sulfur atom from the molybdenum center (Massey & Edmondson, 1970; Edmonson et al., 1972b) which has been suggested to be a persulfide group (Massey & Edmondson, 1970) or a Mo═S group (Bordas et al., 1980; Wahl & Rajagopalan, 1982). No alterations in the FAD or Fe/S centers have been observed for the desulfo-enzyme. The major effect of desulfuration is on the redox and ESR properties of the molybdenum center. The ESR spectrum takes on the characteristics of the "slow" signal (Bray, 1980), and the Mo^{VI}/Mo^V redox couple decreases in potential by 0.035 V (Porras & Palmer, 1982). Since the kinetic properties observed for the native enzyme (Figure 2) which contained about 40% desulfo-enzyme could be influenced by this form, we performed a similar series of flash experiments on the CN$^-$-treated enzyme (which is 100% desulfo). These measurements show that the Fe/S centers in the desulfo-enzyme are reduced in a biphasic manner and at rates which are close to those observed with the native enzyme (data not shown). However, the observed signal intenities are about half those found with unmodified xanthine oxidase. The decay of the deazaflavin radical differs from that found in the presence of the native enzyme in that its rate is independent of enzyme concentration and is considerably slower. The FAD radical is produced rapidly and in amounts similar to that observed with the native enzyme by an analogous direct reaction with the deazaflavin radical. However, an appreciable decay corresponding to its oxidation is observed (Figure 3c), in contrast to what is found with the native enzyme (compare with Figure 3b). The rate of this decay is approximately that of Fe/S$_{II}$ reduction. Taken together, these results suggest that the deazaflavin radical reacts directly with the FAD moiety (intramolecularly) and not with the desulfo-Mo and that the FAD radical produced reduces the Fe/S$_{II}$ center. No indication that the FAD radical reduces Fe/S$_I$ is found. This is consistent with the redox potentials of the two centers (−235 mV vs. −300 mV, respectively). The observed concentration independence of deazaflavin radical decay supports the contention that the desulfo-Mo is not reduced (at least not rapidly) rather than the alternative possibility that it is reduced by the deazaflavin radical but is not able to transfer electrons to the other centers in the enzyme.

The lack of any evidence for the participation of the FAD radical or the desulfo-Mo in the reduction of Fe/S$_I$ leaves only the deazaflavin radical as a possible source of reducing equivalents. When samples of desulfo-xanthine oxidase were partially reduced to a level such that the Fe/S$_{II}$ and FAD moieties were reduced, the transients corresponding to the FAD radical and to the reduction of Fe/S$_{II}$ disappeared. The reduction of Fe/S$_I$, however, was still apparent at a rate similar to that observed with the partially reduced native enzyme. We suggest that this transient is due to a relatively slow direct reduction of Fe/S$_I$ by the deazaflavin radical which proceeds in the absence of functional molybdenum. Since this reaction occurs so slowly, it is difficult to obtain a value for the second-order rate constant, due to the limited range of accessible concentrations.

Kinetic Studies with Enzyme Analogues Modified at the Molybdenum Center. Since the above data were consistent with the molybdenum center serving as one of the entry points for reduction of xanthine oxidase by the deazaflavin radical, it was of interest to modify the molybdenum either to alter its oxidation–reduction potential or to form a stable, reduced form. Recent studies by Hille et al. (1982) and by Barber & Siegel (1983) have shown that arsenite, a known inhibitor of the enzyme, binds to the functional molybdenum center and alters its oxidation–reduction potential. Barber & Siegel (1983) have shown that the potential of the Mo^{VI}/Mo^{V} redox couple upon arsenite complexation is −0.13 V,[3] as compared to −0.3 V (Porras & Palmer, 1982) for the uncomplexed enzyme. Furthermore, they have shown that arsenite binding to the desulfo-enzyme results in only small changes in the Mo^{V} ESR spectrum. Thus, a comparison of the kinetic properties of the arsenite-complexed enzyme with those of the native form should provide information concerning the role of Mo^{V} in the observed intramolecular electron transfer, since it would now be the highest potential center in the enzyme and thus not thermodynamically capable of reducing the other redox centers.

Laser photolysis transients obtained at 590 nm show that, as in the native enzyme and the desulfo-enzyme, the FAD radical is rapidly formed in the arsenite–enzyme complex (data not shown). It differs from the active form, but coincides with the properties of the desulfo form, in that an appreciable subsequent FAD radical oxidation is observed with a rate constant corresponding to Fe/S$_{II}$ reduction. The reduction of Fe/S centers observed at 483 nm proceeds at the same rate as in the native enzyme; however, the amplitude of the reduction is approximately half that of the native enzyme (Figure 6). Since only half of the enzyme is in the arsenite-complexed form, it is possible that this Fe/S center reduction is due to the desulfo-enzyme present and that actually no Fe/S reduction occurs via molybdenum on photolysis of the oxidized arsenite enzyme. This suggestion is consistent with the higher potential Mo^{V} serving as the "electron sink", with no visible color change occurring coincident with its formation.

The optical transient at 483 nm due to the reduction of Fe/S$_I$ is still apparent in the partially reduced arsenite complex; however, the transient due to the reduction of the Fe/S$_{II}$ center has now disappeared (Figure 6). Partial reduction of the arsenite enzyme also results in an approximately 2-fold *increase* in the amplitude of Fe/S$_I$ reduction relative to the oxidized form. This increase in amplitude is not entirely unexpected inasmuch as the high-potential Mo^{VI} is now stoichiometrically converted into Mo^{V} and thus the Fe/S$_I$ center serves as the electron sink. The Mo^{V}/Mo^{IV} redox couple in the arsenite-complexed form is lower in potential than that of the native form (Barber & Siegel, 1983) and thus could serve as a facile reductant for Fe/S$_I$ following reduction of Mo^{V} by the deazaflavin radical. Consistent with this, the rate of deazaflavin radical decay increases 3–4-fold upon partial reduction of the enzyme (data not shown).

It has been known since the work of Massey et al. (1970) that the Mo^{IV} form of xanthine oxidase can be stabilized by complexation with alloxanthine. This allows a form of the enzyme to be studied in which the FAD and Fe/S centers are oxidized while the molybdenum is reduced and thus not able to participate in intramolecular electron transfer. Kinetic results can therefore be obtained in which only the FAD and Fe/S centers are involved. The problem of the existence of functional and nonfunctional (i.e., desulfo) forms of the enzyme was circumvented by using conditions (Edmondson et al., 1972b) such that both forms of the molybdenum center were complexed with alloxanthine.

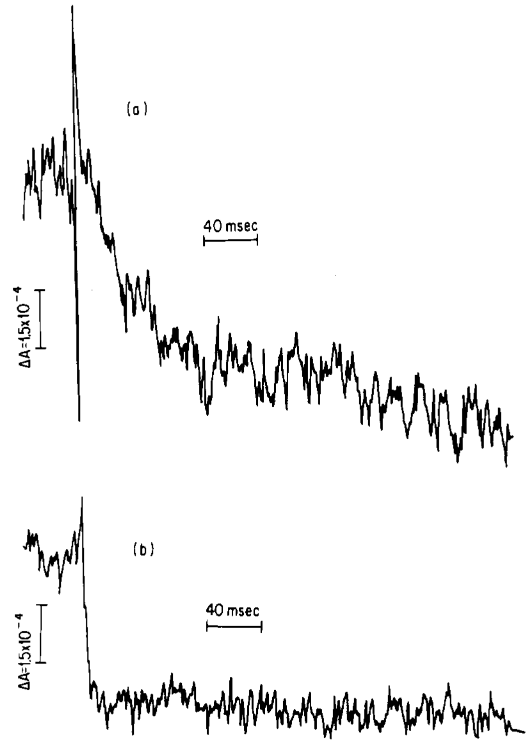

FIGURE 6: Transient absorbance changes observed at 483 nm in arsenite-treated xanthine oxidase. Conditions as in Figure 2. (a) Oxidized; (b) 50% reduced. Sensitivity in (b) is half that in (a) and the same as that in Figure 2.

The kinetic data observed with this form of the enzyme at 590 nm (data not shown) demonstrate that FAD reacts with the deazaflavin radical very rapidly, as found with the other forms of the enzyme, and that the decay of the FAD radical coincides with the reduction of Fe/S$_{II}$. The deazaflavin radical decay was found to be independent of enzyme concentration (Figure 4), as was also observed for the desulfo-enzyme. This is again consistent with the reduction of the functional molybdenum center being responsible for the observed pseudo-first-order decay of the deazaflavin radical (cf. Figure 4) and with FAD reduction being intramolecular. This concentration independence also confirms the previous suggestion that desulfo-Mo^{VI} does not react rapidly with the deazaflavin radical.

The transients observed at 483 nm (data not shown) demonstrate that both Fe/S centers are reduced at rates comparable to those of the native enzyme. The signal amplitudes, however, are again approximately half those found with native xanthine oxidase. Presumably, FAD radical is involved in Fe/S$_{II}$ reduction (its slow decay is observed), and Fe/S$_I$ is reduced via the slow reaction with the deazaflavin radical.

Kinetic Studies of Deflavo-Xanthine Oxidase. In agreement with expectations, no transient absorbance at 590 nm due to FAD radical formation is observed on the reaction of the deazaflavin radical with deflavo-xanthine oxidase. The rate of deazaflavin radical decay is dependent on the enzyme concentration, giving an apparent second-order rate constant

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[3] This is the value reported by using frozen samples to determine the concentration of Mo^{V}. No data at ambient temperature are available.

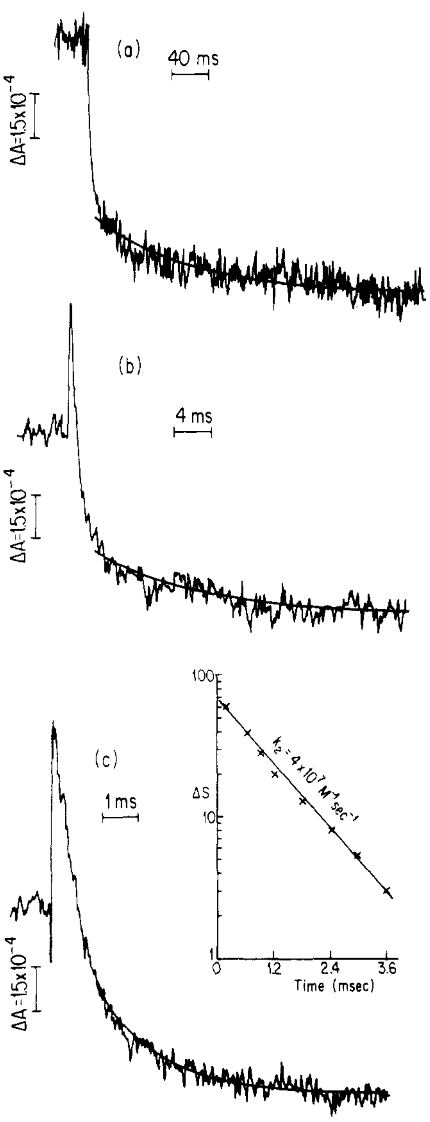


FIGURE 7: Transient absorbance changes observed at 483 nm upon laser flash photolysis of deflavo-xanthine oxidase. Conditions as in Figure 2. The insert in (c) represents a plot of the exponential fit to the decay curve (shown as a superimposed solid line; this is not extended back to zero time for purposes of clarity). (a) 400-ms time scale; (b) 40-ms time scale; (c) 10-ms time scale.

of 3.8×10^7 M^{-1} s^{-1} (Figure 4), a value similar to that observed with the native enzyme (see above) and consistent with the functional molybdenum center being the other reactant in this second-order reaction.

The transients observed at 483 nm (Figure 7) show three discrete components associated with Fe/S center reduction: a very fast component (coincident with deazaflavin radical decay; compare Figure 7b with Figure 2b), a component comparable in rate to that observed for the reduction of Fe/S_I in the native enzyme, and a slow component similar in rate to that observed in native xanthine oxidase for the reduction of Fe/S_{II}. Upon partial reduction of the deflavoenzyme, the slow kinetic component disappears, as expected since the Fe/S_{II} center is now reduced. In addition, the very fast component at first slows down (consistent with the rate of reaction being dependent on the concentration of oxidized Fe/S_{II}) and finally disappears on further reduction, leaving only the component due to Fe/S_I reduction. Thus, we may conclude that Fe/S_{II} reduction occurs *both* via a direct reaction with the deazaflavin radical and via a slow pathway in the deflavoenzyme. The slow component is presumably due to reduction of Fe/S_{II} by the Mo^V generated during the flash by reaction of Mo^{VI} with the deazaflavin radical, as in the other forms of the enzyme. It would appear then that removal of the FAD allows Fe/S_{II} to react directly with the deazaflavin radical. If we assume that all of the enzyme molecules contain reducible Fe/S_{II}, whereas only half contain functional molybdenum, the above second-order rate constant for Mo reduction can be corrected to a value of 7.6×10^7 M^{-1} s^{-1}.

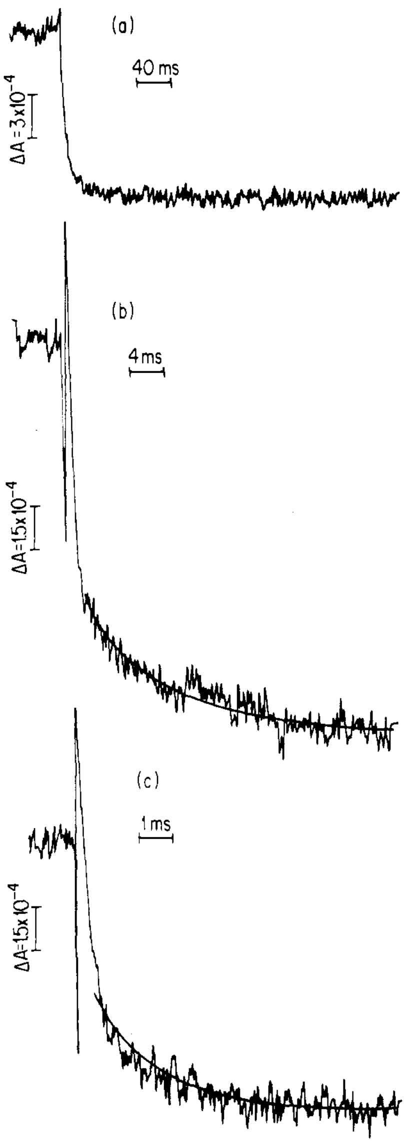


FIGURE 8: Transient absorbance changes observed at 483 nm upon laser flash photolysis of alloxanthine-complexed deflavo-xanthine oxidase. Conditions as in Figure 2. Sensitivity in (a) is half that in (b) and (c) and is the same as that in Figure 2. (a) 400-ms time scale; (b) 40-ms time scale; (c) 10-ms time scale.

Further evidence for this reduction pathway is obtained from flash photolysis of deflavo-xanthine oxidase in which the molybdenum center is trapped as Mo^{IV} via its complexation with alloxanthine. Reaction of this modified form of the enzyme with the deazaflavin radical results in the appearance of only the two rapid components of Fe/S reduction (Figure 8). Thus, Fe/S_{II} again is reduced rapidly by a direct reaction with the deazaflavin radical, and Fe/S_I is reduced somewhat more slowly. This latter reduction also proceeds by (a slower) direct reaction with photochemically generated deazaflavin radical inasmuch as the molybdenum is now nonfunctional as a redox component. In agreement with the proposed direct reaction of deazaflavin radical with each of the Fe/S centers, the rate of decay of deazaflavin radical was proportional to the concentration of enzyme with an apparent second-order rate constant of 6.4×10^7 M^{-1} s^{-1}. Since this does not have to be corrected for the functional enzyme concentration, it can be compared directly to the other rate constant values given above. As expected, the decay rate was found to be dependent on the degree of Fe/S reduction. These observations contrast directly with the observed independence of deazaflavin radical decay upon the concentration of alloxanthine-complexed native enzyme. Thus, removal of the FAD moiety and alteration of the molybdenum center result in a xanthine oxidase form which is still capable of rapid reduction by an external donor via the Fe/S centers.

We have also performed flash experiments on native and desulfo- (CN-treated) enzymes containing an alkylated flavin moiety (Komai & Massey, 1971) which would be incapable of transferring electrons to any of the other redox centers of the enzyme. In the alkylated native enzyme, reduction of both Fe/S centers occurred at rates comparable to those observed in the native enzyme (data not shown). This demonstrates that the rapid reduction of Fe/S_{II} observed in the deflavoenzyme requires more than just a nonfunctional flavin and that molybdenum is capable of serving as a reductant for both Fe/S centers. In addition, partial reduction of the enzyme ($\sim$25%) resulted in a loss of the slow Fe/S component as was found in the native enzyme. In the alkylated desulfo-enzyme, no Fe/S_{II} reduction was observed. However, Fe/S_I reduction occurred at a rate similar to that in the native enzyme, although the amplitude of the signal due to reduction of this center was smaller than in the native enzyme. No FAD transients were found in either the native or the desulfo-enzymes, as expected. These experiments therefore provide further evidence for a direct reduction of Fe/S_I by the deazaflavin radical. They further suggest that no electron transfer from Fe/S_I to Fe/S_{II} is occurring.

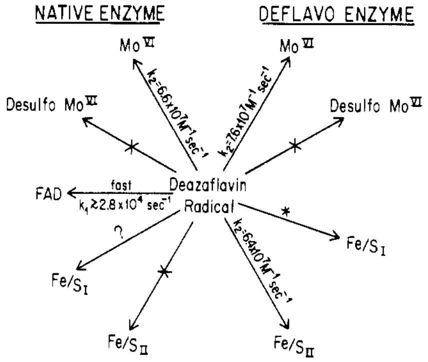
Discussion

The results quoted above clearly demonstrate the utility of the laser photolysis technique is studying the kinetic properties of complex, multifunctional redox enzymes. One of the goals of this study was to provide direct evidence for or against the hypothesis of Olson et al. (1974) that the distribution of reducing equivalents among the various redox centers of xanthine oxidase is the result of a rapid equilibrium governed by the relative redox potentials. The recent publication of these potentials determined at ambient temperature (Porras & Palmer, 1982) was very helpful in our interpretations.

The present kinetic data show that the functional molybdenum center and FAD serve as major reaction sites in the native enzyme for the photochemically generated deazaflavin radical. It is of interest that desulfo-Mo is not kinetically functional with this highly reactive radical, even though thermodynamic considerations would suggest a facile electron transfer. Analogous behavior is also observed with other nonspecific reductants. Thus, it was shown by Edmondson et al. (1972b) that $NaBH_4$ will reduce only the functional enzyme and not the desulfo form.

The FAD moiety of both functional and nonfunctional forms of the enzyme is reduced quite rapidly by the deazaflavin radical. Furthermore, when molybdenum is nonfunctional, a slow reaction of the deazaflavin radical and Fe/S_I apparently occurs. The Fe/S_{II} center is directly reduced by the deazaflavin radical upon removal of the FAD moiety. This change in reactivity suggests an alteration in the environment of this component. No direct structural data have been obtained which would support such an environmental modification. Previous work has shown that the ESR and circular dichroism (CD) properties of the Fe/S centers in the deflavoenzyme are identical with those in the native enzyme (Komai et al., 1969). Furthermore, unpublished studies in our laboratory have shown that the ^1H electron nuclear double resonance (ENDOR) spectra observed for proton couplings to Fe/S_I and Fe/S_{II} are identical for the native and deflavo forms of the enzyme. Thus, if a structural modification of the environment of the Fe/S centers is indeed occurring upon FAD removal, it must be a rather subtle one.

Scheme I: Reactivity of the Deazaflavin Radical with the Redox Centers of Xanthine Oxidase and Deflavo-Xanthine Oxidase[a]



[a] The reaction indicated with the asterisk apparently occurs in the deflavoenzyme, but we are unable to determine a value for the second-order rate constant. It is undoubtedly smaller than the constant for Fe/S_{II} reduction, however. It also occurs in the desulfo form of the enzyme.

Taken together, the kinetic data show that the reactivity of the deazaflavin radical toward the various redox centers of the enzyme is not identical. Such discrimination is, of course, a requirement for the determination of the rates of intramolecular electron transfer among the redox centers of the enzyme. A summary of the reactivity of each of the centers of xanthine oxidase with the deazaflavin radical is shown in Scheme I.

One of the transients observed in the visible spectral region, the formation of the neutral form of the FAD radical, is the simplest to detect, inasmuch as none of the other possible redox species would give rise to a net increase in absorption. The native enzyme (50–60% functional) showed only a small FAD radical decay after its initial formation. In actuality, however, a further, slower reduction of FAD must occur as a result of electron transfer from Mo^V. We suggest that the reason this is not observed is that the FAD radical decays in the desulfo form of the enzyme. Thus, the extent of FAD radical formation as a result of intramolecular transfer from Mo^V must approximately equal that produced upon direct deazaflavin radical reduction and must occur at a rate close to 12 s^{-1}. The use of a fully functional enzyme would have been very helpful in testing this suggestion. However, efforts by each of our laboratories to obtain such a preparation by using the method of Nishino et al. (1981) were unsuccessful. It is also possible, on thermodynamic grounds, that some FAD radical formation occurs via electron transfer from reduced Fe/S_I, although we do not observe this kinetically.

The observed biphasic reduction of the Fe/S centers is due to the individual rates of electron transfer to each of the two centers. The kinetic experiments performed with partially reduced samples of enzyme clearly show that the slow component is due to reduction of Fe/S_{II}, whereas the more rapid component corresponds to Fe/S_I. This is the same in all forms of the enzyme tested, with the exception of the deflavo forms where some fast direct reduction of Fe/S_{II} by the deazaflavin radical occurs. We find no evidence for electron transfer

Table I: Calculated[a] Levels of Each of the Oxidized and Reduced[b] Components of Xanthine Oxidase at pH 7.2 upon One-Electron Reduction on the Basis of Their Respective Redox Potentials

xanthine oxidase form	Mo^{VI}	Mo^{V}	Mo^{IV}	Fe/ $S_{II(ox)}$	Fe/ $S_{II(red)}$	Fe/ $S_{I(ox)}$	Fe/ $S_{I(red)}$	FAD	FADH·	$FADH_2$
native	0.98	0.02	0	0.46	0.54	0.98	0.02	0.81	0.19	0
alloxanthine, native	0	0	1.0[c]	0.45	0.55	0.98	0.02	0.81	0.19	0
deflavo	0.92	0.08	0	0.18	0.82	0.92	0.08	0	0	0
alloxanthine, deflavo	0	0	1.0[c]	0.13	0.87	0.88	0.12	0	0	0

[a] Hille et al. (1981) and Olson et al. (1974). [b] Porras & Palmer (1982). [c] Level of reduction prior to laser photolysis.

occurring between the two Fe/S centers.

It is appropriate at this point to compare the levels of reduction of each of the redox components of xanthine oxidase which are predicted to be formed upon one-electron reduction, on the basis of the relative redox potentials, with those actually observed in the flash photolysis experiments. The equlibrium values listed in Table I show that substantial amounts of reduced Fe/S_{II} should be present, but only trivial amounts of reduced Fe/S_I. This is clearly at variance with the flash photolysis data shown in Figures 2 and 3, in which appreciable amounts of reduced Fe/S_I are formed relative to reduced Fe/S_{II} and to FAD radical at the end of a 400-ms time period following the laser flash. Furthermore, no apparent reoxidation of reduced Fe/S_I is observed on this time scale. These results suggest that the equilibrium distribution of a single electron among the redox centers of xanthine oxidase must occur in times longer than 400 ms. We will return to this again below.

The rate constant for Fe/S_I reduction in the native enzyme (77 s^{-1}) is due to intramolecular electron transfer from Mo^V produced upon reduction of Mo^{VI} by the deazaflavin radical. This rate is faster than that observed in rapid-freeze ESR or in stopped-flow kinetic studies by approximately a factor of 5 (Edmondson et al., 1973). It should be realized, however, that the latter work was done with xanthine as a reductant, and thus, Mo^{IV} was produced upon two-electron reduction.

The rate of reduction of Fe/S_{II} is approximately one-seventh the rate of Fe/S_I reduction. The rate constant for this reaction (12 s^{-1}) is, in fact, comparable to that found for total Fe/S reduction in rapid mixing experiments using xanthine as the reductant. We will return to this point again below. In the laser experiments, intramolecular electron transfer to Fe/S_{II} originates from the FAD radical in the desulfo-enzyme and from both the FAD radical and Mo^V in the functional enzyme. The observed rate is apparently the same, independent of the reducing center.

From the above rate data for reduction of the two Fe/S centers, it is possible to calculate the rate constants for both the forward and reverse reactions with their respective electron donors. We will also assume that electron transfer to FAD from Mo^V proceeds at the same rate as the reduction of Fe/S_{II}. The observed rate constant is the sum of the forward and reverse constants for a simple first-order reaction system

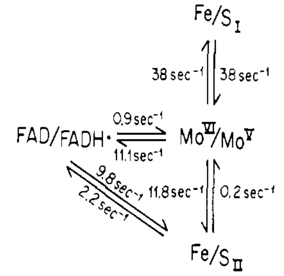
$$k_{obsd} = k_f + k_b \quad (1)$$

and the ratio of the two rate constants is determined by the equilibrium constant for the electron-transfer reaction:

$$K_{eq} = k_f/k_b \quad (2)$$

K_{eq} can be readily calculated from the known redox potentials of the centers involved. The rate constants calculated by using this approach are given in Scheme II. The most rapid rates of electron transfer are those between molybdenum and Fe/S_I. The rate constants for electron transfer between Mo^V and FAD, between reduced Fe/S_{II} and Mo^{VI}, and between the FAD radical and Fe/S_{II} are in the range 10–12 s^{-1}; the remaining rate constants are appreciably slower.

Scheme II: First-Order Rate Constants Calculated for Electron Transfer among the Redox Centers of Native Xanthine Oxidase[a]



[a] These are based on the measured redox potentials (Porras & Palmer, 1982).

It is important to attempt to reconcile the present kinetic data with those observed previously by other rapid kinetic techniques. An apparent inconsistency is immediately evident in that Fe/S_I is reduced approximately 5 times faster than is Fe/S_{II} in the flash experiments, whereas the rapid-freeze ESR data show the two Fe/S centers being reduced at the same rate (Edmondson et al., 1973).[4] This apparent contradiction (if it is not artifactual) can be resolved by the following explanation, which unfortunately cannot be experimentally verified at present for technical reasons. In the case of the rapid mixing experiments, the rate of reduction was followed subsequent to mixing the enzyme with the substrate (xanthine) under anaerobic conditions. In this circumstance, the initial event would involve a two-electron reduction of Mo^{VI} to Mo^{IV}. If the oxidation of Mo^{IV} by either Fe/S_I or Fe/S_{II} is slow (i.e., ca. 12 s^{-1}), the resulting Mo^V would reduce the remaining Fe/S center (either Fe/S_I or Fe/S_{II}) with the rate constants observed in the flash experiments (77 or 12 s^{-1}, respectively). In this situation, both Fe/S centers would be reduced at the same rate since the rate limitation would be due to Fe/S reduction by Mo^{IV}. Alternatively, the Mo^{IV} could also reduce FAD relatively slowly, resulting in Mo^V and FAD radical, which could then function as reductants for Fe/S_I and Fe/S_{II} with the rate constants described above. Here again, the rate limitations would involve Mo^{IV} oxidation, resulting in the same observed rate of Fe/S center reduction. Data presented in this paper suggest that on partial reduction of the arsenite-complexed enzyme, the Mo^{IV} species serves as a facile reductant for Fe/S_I. This form of molybdenum, however, does not function catalytically and thus may have quite different kinetic properties than does the Mo^{IV} form of the functional enzyme. On the other hand, substrates/products bound to the enzyme during catalysis are thought to perturb the potential of the molybdenum and to stabilize the otherwise thermodynamically

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[4] The apparent coincidence of the rates of the reduction of the two Fe/S centers is inconsistent with the rapid equilibrium hypothesis because of the difference in the relative potentials. This is a possible artifact of freezing (Williams-Smith et al., 1977; Palmer & Olson, 1980).

unstable Mo^{IV} (Olson et al., 1974; Palmer & Olson, 1980). It remains for future work to assess the validity of this explanation.

In conclusion, the information contained in this paper clearly demonstrates that the rates of intramolecular electron transfer in native xanthine oxidase are not excessively fast, even on a millisecond time scale. Furthermore, it is evident that the equilibrium levels (i.e., those based on the measured potentials) of the various reduced centers are not completely achieved, at least upon one-electron reduction, at a rate which is faster than catalysis.

Registry No. FAD, 146-14-5; Mo, 7439-98-7; xanthine oxidase, 9002-17-9; 5-deazariboflavin radical, 78548-68-2.

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