Thomassin, H., Nidergang, C., & Mandel, P. (1985) *Biochem. Biophys. Res. Commun. 133*, 654–661.
Thomassin, H., Jacobson, M., Guay, J., Verreault, A., Aboul-ela, N., Menard, L., & Poirier, G. G. (1990) *Nucleic Acids Res. 18*, 4691–4694.
Ueda, K., & Hayaishi, O. (1985) *Annu. Rev. Biochem. 54*, 73–100.
Yoshihara, K., Hashida, T., Tanaka, Y., Ohguchi, H., Yoshihara, H., & Kamiya, T. (1978) *J. Biol. Chem. 253*, 6459–6466.

Kinetic Comparison of Reduction and Intramolecular Electron Transfer in Milk Xanthine Oxidase and Chicken Liver Xanthine Dehydrogenase by Laser Flash Photolysis†

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ABSTRACT: A comparative study using laser flash photolysis of the kinetics of reduction and intramolecular electron transfer among the redox centers of chicken liver xanthine dehydrogenase and of bovine milk xanthine oxidase is described. The photogenerated reductant, 5-deazariboflavin semiquinone, reacts with the dehydrogenase (presumably at the Mo center) in a second-order manner, with a rate constant ($k = 6 \times 10^7$ M^{-1} s^{-1}) similar to that observed with the oxidase [$k = 3 \times 10^7$ M^{-1} s^{-1}; Bhattacharyya et al. (1983) *Biochemistry 22*, 5270–5279]. In the case of the dehydrogenase, neutral FAD radical formation is found to occur by intramolecular electron transfer ($k_{obs} = 1600$ s^{-1}), presumably from the Mo center, whereas with the oxidase the flavin radical forms via a bimolecular process involving direct reduction by the deazaflavin semiquinone ($k = 2 \times 10^8$ M^{-1} s^{-1}). Biphasic rates of Fe/S center reduction are observed with both enzymes, which are due to intramolecular electron transfer ($k_{obs} \approx 100$ s^{-1} and $k_{obs} = 8$–11 s^{-1}). Intramolecular oxidation of the FAD radical in each enzyme occurs with a rate constant comparable to that of the rapid phase of Fe/S center reduction. The methylviologen radical, generated by the reaction of the oxidized viologen with 5-deazariboflavin semiquinone, reacts with both the dehydrogenase and the oxidase in a second-order manner ($k = 7 \times 10^5$ M^{-1} s^{-1} and 4×10^6 M^{-1} s^{-1}, respectively). Alkylation of the FAD centers results in substantial alterations in the kinetics of the reaction of the viologen radical with the oxidase but not with the dehydrogenase. These results suggest that the viologen radical reacts directly with the FAD center in the oxidase but not in the dehydrogenase, as is the case with the deazaflavin radical. The data support the conclusion that the environments of the FAD centers differ in the two enzymes, which is in accord with other studies addressing this problem from a different perspective [Massey et al. (1989) *J. Biol. Chem. 264*, 10567–10573]. In contrast, the rate constants for intramolecular electron transfer among the Mo, FAD, and Fe/S centers in the two enzymes (where they can be determined) are quite similar.

Xanthine oxidase (XO)[1] and xanthine dehydrogenase (XDH) are complex metalloflavoproteins that catalyze the oxidation of xanthine to uric acid and are thought to differ mainly in their electron acceptor specificities (O_2 and NAD^+, respectively). Since the dehydrogenase-to-oxidase interconversion has been demonstrated in enzyme preparations from a number of mammalian sources (Waud & Rajagopalan, 1976; Nakamura & Yamazaki, 1982), it is of interest to compare the properties of both forms in order to provide insights into the structural basis for the alteration in the oxidizing substrate specificity. The current evidence suggests that structural changes around the FAD site are responsible and that differences in the regions containing the Mo and the two Fe_2/S_2 centers are either nonexistent or too small to be detected by spectroscopic approaches (Barber et al., 1980). Recent comparative studies of the FAD environments in bovine XO and in chicken XDH (Massey et al., 1989) have indicated the presence of a negative charge in the flavin-binding site of the dehydrogenase but not the oxidase. Similar conclusions have been reached in studies of rat liver XDH and XO (Saito et al., 1989).

Since the suggestion by Olson et al. (1974) that electron distribution among the Mo, FAD, and the Fe/S I and Fe/S II centers of XO is kinetically rapid relative to catalytic turnover and is in accord with the relative redox potentials of the above centers, a number of experimental approaches have been employed to measure the rates of intramolecular electron

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[1] Abbreviations: XO, bovine milk xanthine oxidase; XDH, chicken liver xanthine dehydrogenase; dRf•, 5-deazariboflavin semiquinone, $MV^{•+}$, methylviologen cation radical.

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transfer among the redox centers of this enzyme. Using laser flash photolysis, in which a substoichiometric level of reducing equivalents in the form of the low potential 5-deazariboflavin semiquinone (dRf$^{\bullet}$) is generated rapidly for reaction with XO, Bhattacharyya et al. (1983) found that the Fe/S centers were reduced in a biphasic manner with observed rate constants of 77 s^{-1} and 12 s^{-1}, with electron entry into the enzyme occurring by reaction of the dRf$^{\bullet}$ with both the Mo and the FAD centers. Subsequent pH-jump stopped-flow experiments on partially reduced XO (Hille & Massey, 1986), in which the relative oxidation–reduction potentials of the redox centers were perturbed by a rapid pH change, demonstrated observed rate constants of electron redistribution among the flavin and Fe/S centers to be 155 s^{-1} at pH 6 and 330 s^{-1} at pH 8.5, which suggests that the rates of electron distribution are at least 10-fold greater than k_{cat}. Pulse radiolysis studies on XO (Anderson et al. 1986) demonstrated that the $CO_2^{-\bullet}$ radical reacted with the FAD center and that the resulting neutral FAD radical (FADH$^{\bullet}$) reduced the Fe/S II center with an observed rate constant of $\approx$290 s^{-1}. Additional evidence was presented for the reduction of a disulfide bond, apparently identical with the one reduced by dithionite (Hille & Massey, 1982), by the radiolytically generated radicals. Anderson et al. (1986) have suggested that a similar event may occur in the reaction of dRf$^{\bullet}$ with XO and that this could be responsible for the slower rates of electron transfer observed by Bhattacharyya et al. (1983), as a consequence of structural changes in the enzyme caused by disulfide bond reduction. As a test of the validity of this suggestion, Edmondson et al. (1987) showed that the rates of Fe/S reduction observed on laser flash photolysis of a preparation of xanthine oxidase in which the dithionite-reducible disulfide bond had been previously reduced and carboxymethylated were identical with those observed with the native enzyme. Thus, reduction of the disulfide bond by the dRf$^{\bullet}$, if it does occur during the laser photolysis experiments, has no influence on the observed kinetics of electron transfer. Additionally, Edmondson et al. (1987) found that dRf$^{\bullet}$ reduced the FAD in XO to its neutral semiquinone form with a second-order rate constant of 2×10^8 M^{-1} s^{-1} and that the FAD radical subsequently reduced Fe/S II with an observed rate constant of 100 s^{-1}. This latter rate constant was found to be the same at pH 8.5 and 7.0 and is about one-third of the value reported by Anderson et al. (1986) using the pulse radiolysis technique. A possible explanation for the differences in rate constants obtained by the two approaches is that only a single reducing equivalent is introduced into the enzyme with the laser flash technique, whereas multiple electron equivalents are introduced with the pulse radiolysis method. Furthermore, in the pulse radiolysis experiments, much stronger reductant species are generated, which could produce nonspecific effects. In the case of the pH-jump experiments, changes in the electrostatic charge on the protein caused by titration of acid/base groups could lead to structural perturbations that influence the observed kinetics.

A recent study (D'Ardenne & Edmondson, 1990) estimated the microscopic rate constants for C–H bond cleavage in xanthine [and therefore Mo(VI) reduction to Mo(IV)] and found the minimal value for XO to be 1400 s^{-1} and for XDH to be 200 s^{-1}. This difference is most probably due to the diverse origins of the two enzymes (bovine milk and chicken liver) rather than to their substrate specificities for oxidants. These results show that in each case electrons are introduced to the Mo center from the substrate rapidly relative to k_{cat} and that a considerable difference exists between the two enzymes as regards the rate constant for this process. Thus, a comparative study of the rates of the electron transfer processes occurring subsequent to the initial reduction of Mo in XO and XDH is of interest. Furthermore, other information points to significant differences in the reactivity of the FAD centers in these two enzymes (Barber et al., 1980; Nishino et al., 1989), and a kinetic comparison, such as described in this paper, may provide some unique insights into their relative properties. The results shown here document that significant differences exist in the kinetic accessibilities of the FAD centers of XO and XDH to exogenous reductants, whereas the rate constants for Mo reduction by dRf$^{\bullet}$ and for electron transfer from FADH$^{\bullet}$ to Fe/S II are quite similar for both enzymes. In the case of the dehydrogenase, we have been able to directly measure the rate constant for intramolecular electron transfer to the FAD center (presumably from the Mo center). The relationship of these results to existing data in the literature, and to the catalytic mechanisms of these enzymes, is discussed.

Materials and Methods

Xanthine oxidase was purified from fresh unpasteurized bovine milk (University of Georgia dairy) according to the procedure described by Massey et al. (1969). Typical preparations exhibited $\approx$60% functionality due to the presence of desulfo-XO (Edmondson et al., 1972b). Xanthine dehydrogenase was purified from fresh chicken liver by the procedure (with only minor modifications) described by Rajagopalan and Handler (1967). Functionality of XDH preparations ranged from 70% to 80%.

FAD-alkylated XO was prepared according to the procedure of Komai and Massey (1971), with enzyme samples reduced by dithionite and with iodoacetamide as the alkylating agent. Samples of XO in which a dithionite-reducible disulfide bond is reduced and alkylated with iodoacetate were prepared as described by Hille and Massey (1982). FAD-alkylated XO exhibited <5% xanthine oxidase activity as compared to the untreated enzyme, whereas the oxidase activity of the iodoacetate-treated enzyme was essentially unaffected, in agreement with the results of Hille and Massey (1982). FAD-alkylated XDH was prepared by anaerobic incubation of dithionite-reduced XDH with excess iodoacetate at room temperature until >90% of the xanthine-NAD^+ oxidoreductase activity was lost (>5 h). Excess reagents were removed by dialysis, with precautions taken to protect the sample from light, which is known to slowly effect dealkylation of FAD-alkylated XO (Komai & Massey, 1971).

Laser flash photolysis experiments were carried out as described previously (Bhattacharyya et al., 1983), although the optical measurement system used was considerably improved over the earlier version, allowing more sensitive detection of transient absorbing species and providing better time resolution. Optical excitation of the system generated the neutral semiquinone of 5-deazariboflavin, via hydrogen-atom abstraction by the flavin triplet state from the sacrificial donor (usually EDTA) (Edmondson et al., 1972a). Protein concentrations were generally at least one order of magnitude larger than the dRf$^{\bullet}$ concentration produced by the flash (<1 μM). Thus, no more than a single electron was deposited into each protein molecule during a measurement, and the concentration of reduced protein within the volume of the sample cuvette that was subject to laser irradiation (approximately 1% of the total volume) was less than 1 μM. The kinetic transients shown below represent the sum of 5–10 laser flashes, collected by signal averaging. Absorbance changes are given in arbitrary units, inasmuch as absolute values depend on a variety of experimental parameters, such as deazaflavin concentration, protein concentration, laser intensity, etc. The

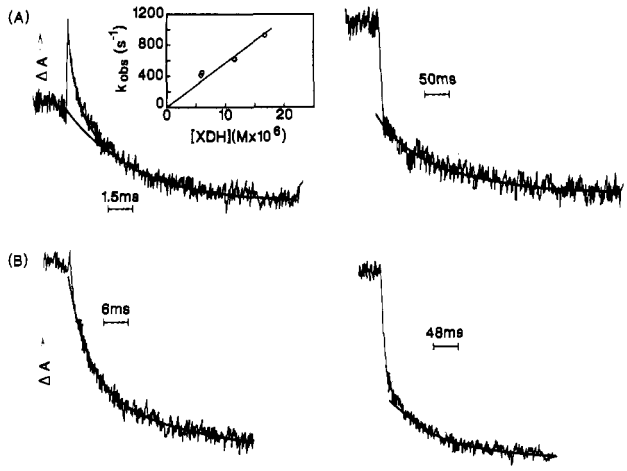

FIGURE 1: Transients obtained at 483 nm upon laser flash photolysis of xanthine dehydrogenase and xanthine oxidase. (A) Xanthine dehydrogenase (17.5 μM) in 0.1 M phosphate buffer, pH 7.8, containing 120 μM deazariboflavin and 20 mM EDTA. Solid curves correspond to single-exponential fits to various portions of the decay curves. (Inset) Protein concentration dependence of the observed rate constant for the initial portion of the absorbance decay, corresponding to disappearance of deazariboflavin semiquinone. (B) Xanthine oxidase (20 μM) in 0.02 M phosphate buffer, pH 7.2, containing 70 μM deazariboflavin and 20 mM EDTA. Solid curves correspond to single-exponential fits to various portions of the decay curves.

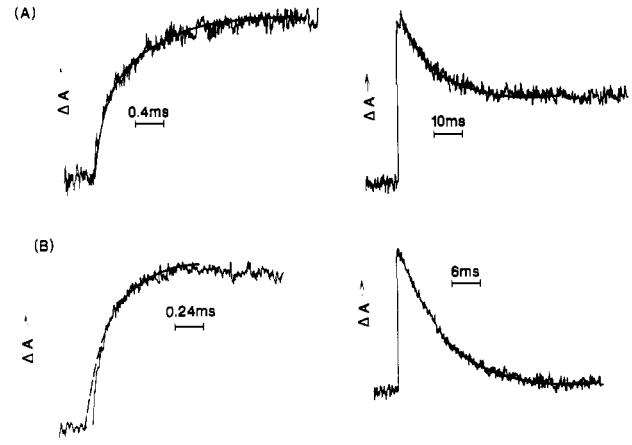

FIGURE 2: Transients obtained at 600–620 nm upon laser flash photolysis of xanthine dehydrogenase and xanthine oxidase. (A) Xanthine dehydrogenase (12.2 μM); buffer conditions were as described in the legend to Figure 1. Solid curves correspond to single-exponential fits to the data. The monitoring wavelength was 600 nm. (B) Xanthine oxidase (20 μM); buffer conditions were as described in the legend to Figure 1, except that the deazaflavin concentration was 130 μM. Solid curves correspond to single-exponential fits to the data. The monitoring wavelength was 620 nm.

analyses presented below are not dependent upon a knowledge of absolute concentrations.

In these experiments, protein reduction was generally in competition with dRf$^{\bullet}$ disproportionation. At the protein concentrations used here, this resulted in an uncertainty of $\leq$20% in the determination of second-order rate constants for protein reduction [cf. Bhattacharyya et al. (1991) for further discussion]. In some experiments, 2 mM methylviologen (MV^{2+}) was present in the buffer. This acted to scavenge all of the dRf$^{\bullet}$ produced by the laser flash prior to reaction with the protein, forming methylviologen radical ($MV^{\bullet+}$), which then served as the reductant of the protein.

Analysis of the kinetic transients was carried out by fitting portions of the decay curve to single-exponential functions (see, for example, Figure 1). Although computer deconvolution can in principle be applied to these data, it was our experience that, as a consequence of the multiphasic nature of most of the transients, this gave less accurate results than the method used. When the kinetic phases were widely separated, computer fitting gave results that were in good agreement with the hand fits.

In order to determine whether or not a given transient absorbance change corresponded to an intermolecular or an intramolecular event, the dependence of the observed rate constant on protein concentration was measured. This was generally done over a range of approximately 5–40 μM. Second-order rate constants were calculated from such measurements, where applicable.

RESULTS

XO and XDH Reduction by 5-Deazariboflavin Semiquinone. Kinetic transients monitored at 483 nm correspond to the formation of dRf$^{\bullet}$ (absorbance increase) and the reduction of Fe/S centers in the enzyme [absorbance decrease; this is an isosbestic point for FAD reduction, and changes due to Mo are generally unobservable; cf. Bhattacharyya et al. (1983) and Edmondson et al. (1987)]. Figure 1 shows that the transient absorbance changes obtained at this wavelength upon laser photolysis of XO and XDH are quite similar to one another. As can be seen in Figure 1A, an initial rapid increase in absorbance is observed with XDH, due to dRf$^{\bullet}$ formation. This is followed by a biphasic absorbance decrease, which is due to reoxidation of dRf$^{\bullet}$ and reduction of the protein Fe/S centers. A deconvolution of this transient is shown by the superimposed solid lines, which correspond to single-exponential fits to the data. Variation of the enzyme concentration demonstrates that only the initial faster transient absorbance decrease is dependent upon protein concentration, and a second-order rate constant of 6×10^7 M^{-1} s^{-1} was obtained (insert to Figure 1A). Inasmuch as no net absorbance change is associated with this process (i.e., the transient returns to the preflash baseline), we assign it to the reduction of the Mo center of the enzyme by dRf$^{\bullet}$. This is analogous to what was previously observed with XO, for which a similar second-order rate constant of 3×10^7 M^{-1} s^{-1} was obtained (Bhattacharyya et al., 1983).

The net decrease in absorbance at 483 nm follows biphasic kinetics for both XDH and XO (Figure 1A,B). The rate constants corresponding to these two phases of Fe/S reduction are separated by approximately one order of magnitude and hence are readily distinguishable. Both rate processes were found to be independent of protein concentration, and consequently can be assigned to intramolecular electron transfer to the Fe/S centers from the other cofactors of the enzyme. The fast phase of Fe/S reduction with XDH exhibits an observed rate constant of 110 s^{-1}, which is comparable to that obtained with XO (k_{obs} = 100 s^{-1}; Edmondson et al., 1987). The slow phase of Fe/S reduction has an observed rate constant of 8 s^{-1} with XDH and 11 s^{-1} with XO (Edmondson et al., 1987).

The redox state of the FAD cofactor is conveniently monitored at wavelengths greater than 600 nm. At neutral pH values, both enzymes produce the neutral (blue) form of the FAD radical, which exhibits a broad absorption band in this region. None of the other redox centers contribute an increase in absorbance on reduction in this portion of the spectrum. As illustrated in Figure 2, laser photolysis results in a monoexponential increase in absorbance with both XDH and XO. With XDH, k_{obs} for this absorbance increase was found to be independent of protein concentration (k_{obs} = 1600 $\pm$ 200 s^{-1}) and hence corresponds to an intramolecular process. Inasmuch as the transients associated with the Fe/S centers in XDH have

rate constants much smaller than 1600 s^{-1}, the reduction of FAD to the semiquinone form must have occurred as a result of electron transfer from the Mo center. This is not the case with XO, for which second-order kinetics are obtained (i.e., protein concentration dependent), with a rate constant of 2×10^8 M^{-1} s^{-1} (Bhattacharyya et al., 1983; Edmondson et al., 1987).

At longer times, the absorbance change at 600 nm is found to decrease in a monoexponential fashion for each enzyme (Figure 2). In both cases, this decrease is independent of protein concentration and therefore intramolecular. The observed rate constant for XO was found to be 118 s^{-1} (Edmondson et al., 1987), whereas that for XDH is 79 s^{-1}. Inasmuch as the Fe/S II centers in XO and XDH have the highest redox potentials of the various cofactors (Porras & Palmer, 1982; Barber et al., 1980; Schopfer et al., 1988), it seems reasonable to assign these transients to electron transfer from $FADH^{\bullet}$ to Fe/S II. In support of this assignment, the observed rate constants for $FADH^{\bullet}$ reoxidation and the fast phase of Fe/S reduction (see above) are of comparable magnitudes, although in the case of XDH the Fe/S rate constant is slightly, but significantly, larger (110 vs 79 s^{-1}). We will return to this again below.

With XDH, the absorbance at 600 nm does not decay completely to the preflash baseline, whereas it does so in the case of XO (Figure 2). This indicates that approximately one-half of the $FADH^{\bullet}$ remains in XDH after electron equilibration, whereas virtually all of the $FADH^{\bullet}$ was reoxidized in XO. These results are consistent with the observations that the redox potentials of the Fe/S II center and the FAD are approximately equal in the case of XDH (Schopfer et al., 1988), whereas the potential of the Fe/S II center in XO is approximately 100 mV higher than that of FAD (Porras & Palmer, 1982).

The amplitude of the initial increase in absorbance at 600 nm due to the formation of $FADH^{\bullet}$ in XDH was found to vary from one enzyme preparation to another, as was the case for the fast and slow Fe/S signals as noted above. In general, maximal fast Fe/S signals are accompanied by smaller $FADH^{\bullet}$ signals. However, the rate constants and the extent of $FADH^{\bullet}$ reoxidation are not altered.

XO and XDH Reduction by Methylviologen Radical. $MV^{\bullet+}$ was generated by the laser flash via the oxidation of $dRf^{\bullet}$, which in this case was formed with 20 mM methionine as the primary electron donor. As noted previously, the reduction of MV^{2+} by $dRf^{\bullet}$ is much faster (more than two orders of magnitude) than enzyme reduction under the conditions of these experiments (Edmondson et al., 1987), and the viologen radical is found to be stable for at least several seconds under anaerobic conditions in the absence of enzyme. As is shown in Figure 3, upon addition of XDH or XO to the reaction mixture, the formation of $MV^{\bullet+}$ (initial absorbance increase at 620 nm) was followed by a monoexponential absorbance decay due to the oxidation of this radical species by the protein. For both enzymes, the observed rate constants were dependent on the protein concentration and were found to be the same as that for the reduction of the Fe/S centers measured at 483 nm (see inserts to Figure 3; these data refer to the fast phase of Fe/S reduction in the case of XO; see below). The second-order rate constants determined are 7×10^5 M^{-1} s^{-1} for XDH and 4×10^6 M^{-1} s^{-1} for XO. It is interesting to note that these reactions are much slower than the corresponding processes with $dRf^{\bullet}$; why this is so is not immediately evident.

With XO, Fe/S reduction was again found to be biphasic, with a slow phase that is independent of protein concentration

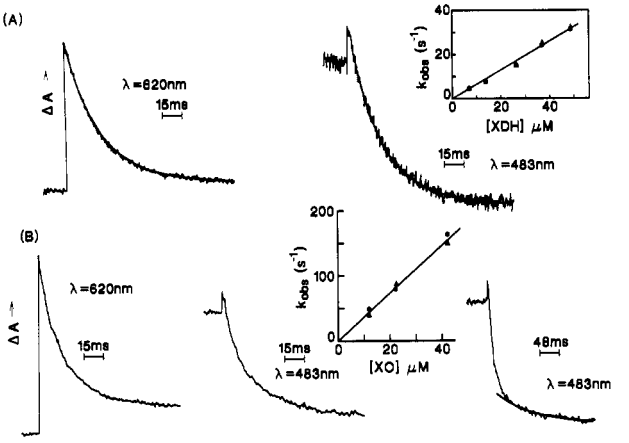

FIGURE 3: Transients obtained at 620 nm and 483 nm upon laser flash photolysis of xanthine dehydrogenase and xanthine oxidase in the presence of methylviologen. (A) Xanthine dehydrogenase (18.6 μM); buffer conditions were as described in the legend to Figure 1, except that 5 mM methylviologen was added. Solid curves correspond to single-exponential fits to the data. (Inset) Protein concentration dependence of the observed rate constants measured at 483 nm (closed triangles) and at 620 nm (open circles). The second-order rate constant calculated from this plot is 7×10^5 M^{-1} s^{-1}. (B) Xanthine oxidase (20 μM); buffer conditions were as described in the legend to Figure 1, except that 2 mM methylviologen was added. The solid curve corresponds to a single exponential fit to a portion of the decay curve. (Inset) Protein concentration dependence of observed rate constants measured at 483 nm (closed circles) and at 620 nm (closed triangles).

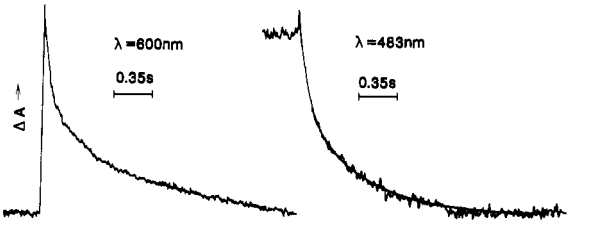

FIGURE 4: Transients obtained at 483 nm and 600 nm for FAD-alkylated xanthine oxidase (40 μM) in the presence of methylviologen. Buffer conditions were as described in the legend to Figure 3B. This sample of xanthine oxidase had also had its disulfide bond reduced and carboxymethylated [cf. Edmondson et al. (1987)]. The solid curve corresponds to a single-exponential fit to latter portion of decay curve.

having the same observed rate constant (11 s^{-1}) as was obtained in the absence of MV^{2+}. In contrast, no concentration-independent slow phase of Fe/S reduction was found upon $MV^{\bullet+}$ reduction of XDH.

Alkylation of the FAD center of XO with iodoacetamide (Komai & Massey, 1971) markedly alters the kinetics of both $MV^{\bullet+}$ oxidation and of Fe/S reduction (Edmondson et al., 1987). With this modified form of the enzyme, $MV^{\bullet+}$ reoxidation is biphasic (Figure 4), with both phases being slower than with the native enzyme and exhibiting second-order behavior (k values are 3×10^5 M^{-1} s^{-1} and 5×10^4 M^{-1} s^{-1}, respectively; data not shown). We interpret these results with XO as indicating that, in the unmodified enzyme, $MV^{\bullet+}$ reacts directly with the FAD center in a rate-determining second-order reaction. When this reaction is prevented by FAD alkylation, $MV^{\bullet+}$ reacts in a slower second-order reaction with both Fe/S centers in the enzyme. We cannot, of course, rule out the possibility that alkylation results in structural changes in XO that modify the reactivities of the Fe/S centers. This possibility is considered unlikely since XO preparations treated with iodoacetate according to the procedure of Hille and Massey (1982) (in which the FAD center is not alkylated whereas thiol groups are) retains the same reactivity with $MV^{\bullet+}$ as the untreated enzyme (Edmondson et al., 1987). The fact that biphasic second-order kinetics for Fe/S reduction are

not observed in the native enzyme also argues against this possibility.

Alkylation of the reduced FAD center of XDH, in contrast to XO, requires more than 5 h of incubation to achieve >90% reaction as monitored by the xanthine-NAD^+ oxidoreductase assay. Massey et al. (1989) reported only marginal FAD alkylation in chicken liver XDH after a 70-min incubation time. FAD-alkylated XDH exhibits the same reactivity with $MV^{\bullet+}$ as observed with the native enzyme (data not shown), and, therefore, we conclude that the FAD center in XDH is unreactive with $MV^{\bullet+}$ (in contrast with XO). Therefore, $MV^{\bullet+}$ probably reduces XDH via the Fe/S centers in a bimolecular manner. The fact that the kinetics of this process are unchanged upon FAD alkylation indicates that this modification does not result in significant structural alterations in the vicinity of the Fe/S sites.

The use of the positively charged methylviologen radical as a reductant allows the use of ionic strength variation to determine the sign of the electrostatic charge at the electron transfer site on the protein. When this was done with XDH, we observed an approximately 5-fold increase in the second-order rate constant for Fe/S center reduction on increasing the ionic strength of the medium from $\mu = 0.06$ M to $\mu = 1.8$ M, indicating that a positive charge is located at or near the Fe/S center (data not shown). Little or no effect of ionic strength over the same range is observed in the reaction of $MV^{\bullet+}$ with XO. We also investigated the effect of ionic strength on the kinetics of reduction of FAD-alkylated XO, inasmuch as direct Fe/S center reduction presumably occurred with this species (see above). However, in this case, we also found no appreciable ionic strength dependence of the reaction rate. This could be a consequence of differences in protein structure in the vicinity of the Fe/S centers in the two enzymes.

Discussion

Kinetic evidence is presented in this paper, with $dRf^{\bullet}$ and $MV^{\bullet+}$ as reductants, for differences in the accessibilities of the FAD centers of XO and of XDH. Thus, both reductants are able to transfer reducing equivalents directly to the FAD center of XO but not to the FAD center of XDH. In a comparative study of these two enzymes using reconstitution of the respective deflavoenzymes with FAD analogues, Massey et al. (1989) demonstrated that the 8 position of the flavin ring is exposed to solvent in both XO and XDH but that the 6 position is significantly less exposed in XDH than in XO. Further studies demonstrated that a strong negative charge is at the flavin-binding site in XDH but not in XO (Massey et al., 1989). Similar results have also been obtained for the oxidase and dehydrogenase forms of rat liver XDH (Saito et al., 1989). Thus, the present kinetic results are consistent with the earlier work.

Further evidence for differences in the reactivities of the respective FAD centers in these two enzymes is the ready alkylation of the reduced FAD in XO by iodoacetamide (Komai & Massey, 1981), whereas in XDH this reaction occurs quite slowly (this paper; Massey et al., 1989). These differences are no doubt a consequence of the proteolytic cleavage and/or thiol oxidation that is believed to convert milk XDH to the oxidase form (Nakamura & Yamazaki, 1982). The higher accessibility of the FAD center in XO may also correlate with the low value for the pK of the FAD semiquinone form, which has been reported to be approximately 8.8; this is to be compared with a pK of 8.4 for free flavins (Draper & Ingraham, 1968). To our knowledge, no ionization of the neutral FAD semiquinone has been observed in XDH in the accessible pH range.

As noted above, the intramolecular reduction of the Fe/S centers of XO and XDH displays biphasic kinetics. In the case of XO, the observed rate constant for the fast phase is closely comparable in value to that for the decay of the $FADH^{\bullet}$ species (100 s^{-1} vs 118 s^{-1}), whereas for XDH the Fe/S reduction rate constant is significantly larger (110 s^{-1} vs 79 s^{-1}). This latter result could be a consequence of a kinetic contribution from intramolecular electron transfer between Mo(V) and one of the Fe/S centers [Fe/S(II)?], occurring with a somewhat larger rate constant (perhaps on the order of 160 s^{-1}). We have previously concluded that Fe/S reduction by Mo(V) also occurs in XO, with an observed rate constant comparable to that for $FADH^{\bullet}$ reoxidation, i.e., 100 s^{-1} (Bhattacharyya et al., 1983; Edmondson et al., 1987).

The slow kinetic phase of Fe/S reduction, first reported in XO by Bhattacharyya et al. (1983) and later confirmed by Edmondson et al. (1987) as well as in XDH by the studies presented here when $dRf^{\bullet}$ was the reductant, has been the subject of some controversy. A kinetic process having a similar rate constant has been previously found in a stopped-flow investigation of the reduction of XDH by NADH at 4 °C (Schopfer et al., 1988). In contrast, such a slow transient has not been found in stopped-flow, pH-jump, or pulse radiolysis experiments with XO (Hille & Massey, 1986; Anderson et al., 1986), although it must be noted that these approaches correspond to rather different kinds of experiments. The fact that this slow Fe/S reduction reaction is not observed in XDH when the source of electrons is $MV^{\bullet+}$ may be due to the fact that $MV^{\bullet+}$ reacts directly with the Fe/S centers. In instances (for either enzyme) where this slow Fe/S reduction is observed in laser photolysis, the source of reducing equivalents is intramolecular, i.e., from the other redox centers in the enzyme. The enzymic significance of this transient must be considered uncertain at the present time. It is clearly slower than turnover in both enzymes [cf. D'Ardenne and Edmondson (1990)] and could reflect the presence of a small fraction (20% or less) of protein that has been structurally altered, perhaps by proteolysis. One possibility, suggested by Hille and Massey (1986), is that this slow reduction is a consequence of enzymic oxidation of an aldehyde product of the primary donor used in these experiments, i.e., glyoxylate in the case of EDTA (Armstrong et al., 1982). Indeed, glyoxylate can serve as a substrate for XO. However, control experiments in this laboratory show that the rate of turnover of this aldehyde at concentrations comparable to those that would be generated by the laser pulse during photolysis (<1 μM) is too slow to account for the observed rate constant of the slow phase of Fe/S reduction. In this context, we have noted that the relative contributions of these two phases of Fe/S reduction varies over a range of approximately 2-fold from one enzyme preparation to another [cf. Bhattacharyya et al. (1983)]. In general, those preparations obtained from the freshest chicken livers in the case of XDH had the smallest proportion of the slow phase [although there was no apparent correlation with percent functionality as defined in the literature, cf. Edmondson et al. (1972b)]. We have also observed that XDH preparations obtained from stored livers had a greater content of proteolytically cleaved enzyme (Coughlan et al., 1979), as determined by SDS gel electrophoresis. In some preliminary experiments in which our XDH preparations were treated with trypsin, we were unable to demonstrate any correlation between extent of proteolysis by this enzyme and the kinetic pattern of Fe/S reduction. Still another possible explanation for the slow phase of Fe/S reduction is that it represents the thermodynamically unfavorable reduction of the relatively low potential Fe/S I

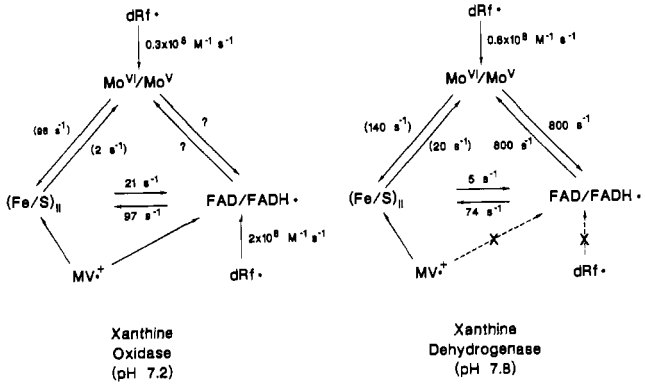

FIGURE 5: Comparison of the kinetic properties of xanthine dehydrogenase and xanthine oxidase as determined by laser flash photolysis. The numbers refer to rate constants as described in text; those in parenthesis correspond to estimated values.

center by Fe/S II. Further work is needed to distinguish between these various possibilities.

A comparison between the microscopic rate constants obtained for XDH and XO in this and the previous investigations (Bhattacharyya et al., 1983; Edmondson et al., 1987) is given in Figure 5. The values for XO were obtained from the room temperature redox potentials determined by Porras and Palmer (1982), by using the fact that for a reversible first-order process the observed rate constant is equal to the sum of the forward and reverse microscopic rate constants. In the case of XDH, room temperature E_m values are not available, and thus we have used the results obtained by Barber et al. (1980) at low temperatures, along with the ordering of the redox potentials deduced at room temperature by anaerobic titrations (Schopfer et al., 1988). It is quite clear from the scheme in Figure 5 that, with the exception of the accessibility of the FAD sites, the two enzymes exhibit quantitatively very similar rate constants for intramolecular electron transfer. It is also important to note that, in the case of XDH, the most rapid intramolecular process involves electron transfer between the Mo(VI)/Mo(V) couple and the FAD/FADH· couple (unfortunately, due to the accessibility of the FAD center to direct reduction, we are unable to determine rate constants for this process in XO). This would be sensible from a catalytic point of view, inasmuch as Mo represents the entry point for electrons and FAD represents the exit point. It is interesting that no spectroscopic indications for Mo/FAD interactions have been observed, although evidence for such an interaction has been found for Mo and Fe/S and for Fe/S I and Fe/S II (Barber et al., 1982). The structural basis for this is unclear at present and may reflect different requirements for electron transfer and magnetic dipole interactions. Another point to be noted is that the present experiments refer only to a one-electron reduced form of the enzyme, and it is certainly possible that the intramolecular rate constants might be different for more highly reduced species. This aspect deserves further investigation.

REFERENCES

Anderson, R. F., Hille, R., & Massey, V. (1986) *J. Biol. Chem. 261*, 4363–4382.

Amstrong, J. S., Hemmerich, P., & Traber, R. (1882) *Photochem. Photobiol. 35*, 747–751.

Barber, M. J., Coughlan, M. P., Kanda, M., & Rajagopalan, K. V. (1980) *Arch. Biochem. Biophys. 201*, 468–475.

Barber, M. J., Salerno, J. C., & Siegel, L. M. (1982) *Biochemistry 21*, 1648–1656.

Bhattacharyya, A. K., Tollin, G., Davis, M., & Edmondson, D. E. (1983) *Biochemistry 22*, 5270–5279.

Bhattacharyya, A. K., Lipka, J. L., Waskell, L., & Tollin, G. (1991) *Biochemistry 30*, 759–765.

Coughlan, M. P., Betcher-Lange, S. L., & Rajagopalan, K. V. (1979) *J. Biol. Chem. 254*, 10694–10699.

D'Ardenne, S. C., & Edmondson, D. E. (1990) *Biochemistry 29*, 9046–9052.

Draper, R. D., & Ingraham, L. I. (1968) *Arch. Biochem. Biophys. 125*, 802–808.

Edmondson, D. E., Barman, B., & Tollin, G. (1972a) *Biochemistry 11*, 1133–1138.

Edmondson, D. E., Massey, V., Palmer, G., Beacham, L. M., & Elion, G. B. (1972b) *J. Biol. Chem. 247*, 1597–1604.

Edmondson, D. E., Hazzard, J. T., & Tollin, G. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 403–408, W. deGruyter, Berlin.

Hille, R., & Massey, V. (1982) *J. Biol. Chem. 257*, 8898–8908.

Hille, R., & Massey, V. (1986) *J. Biol. Chem. 261*, 1241–1247.

Komai, H., & Massey, V. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 399–420, University Park Press, Baltimore, MD.

Massey, V., Brumby, P. E., Komai, H., & Palmer, G. (1969) *J. Biol. Chem. 244*, 1682–1691.

Massey, V., Schopfer, L. M., Nishino, T., & Nishino, T. (1989) *J. Biol. Chem. 264*, 10567–10573.

Nakamura, M., & Yamazaki, I. (1982) *J. Biochem. (Tokyo) 92*, 1279–1286.

Nishino, T., Nishino, T., Schopfer, L. M., & Massey, V. (1989) *J. Biol. Chem. 264*, 2518–2527.

Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974) *J. Biol. Chem. 249*, 4363–4382.

Porras, A. G., & Palmer, G. (1982) *J. Biol. Chem. 257*, 11617–11626.

Rajagopalan, K. V., & Handler, P. (1967) *J. Biol. Chem. 242*, 4097–4107.

Saito, T., Nishino, T., & Massey, V. (1989) *J. Biol. Chem. 264*, 15930–15935.

Schopfer, L. M., Massey, V., & Nishino, T. (1988) *J. Biol. Chem. 263*, 13528–13538.

Waud, W. R., & Rajagopalan, K. V. (1976) *Arch. Biochem. Biophys. 172*, 365–379.