Differences in Protein Structure of Xanthine Dehydrogenase and Xanthine Oxidase Revealed by Reconstitution with Flavin Active Site Probes*

Vincent Massey† and Lawrence M. Schopfer
From the Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

Takeshi Nishino and Tomoko Nishino
From the Department of Biochemistry, Yokohama City University School of Medicine, Yokohama 236, Japan

The native flavin, FAD, was removed from chicken liver xanthine dehydrogenase and milk xanthine oxidase by incubation with CaCl₂. The deflavoenzymes, still retaining their molybdopterin and iron-sulfur prosthetic groups, were reconstituted with a series of FAD derivatives containing chemically reactive or environmentally sensitive substituents in the isoaizoxazine ring system. The existence of enzymes containing these artificial flavins were all catalytically active. With both the chicken liver dehydrogenase and the milk oxidase, the flavin 8-position was found to be freely accessible to solvent. The flavin 6-position was also freely accessible to solvent in milk xanthine oxidase, but was significantly less exposed to solvent in the chicken liver dehydrogenase.

 Pronounced differences in protein structure surrounding the bound flavin were indicated by the spectral properties of the two enzymes reconstituted with flavins containing ionizable —OH or —SH substituents at the flavin 6- or 8-positions. Milk xanthine oxidase either displayed no preference for binding of the neutral or anionic flavin (8-OH-FAD) or a slight preference for the anionic form of the flavin (6-hydroxy-FAD, 6-mercapto-FAD, and possibly 8-mercapto-FAD). On the other hand, the chicken liver dehydrogenase had a dramatic preference for binding the neutral (protonated) forms of all four flavins, perturbing the pK of the ionizable substituent ≥4 pH units. These results imply the existence of a strong negative charge in the flavin binding site of the dehydrogenase, which is absent in the oxidase.

In the first paper of this series, we have described rapid reaction kinetics studies on the reduction of chicken liver xanthine dehydrogenase by xanthine and reduced pyridine nucleotides, as well as the reoxidation of the reduced enzyme by oxidized pyridine nucleotides (Schopfer et al., 1988). In the second paper, we concentrated mainly on the catalytic reaction of the enzyme with molecular oxygen as acceptor, including both rapid reaction and steady state kinetics studies (Nishino et al., 1989a). In both of these publications, we drew attention to the similarities and differences found between the chicken liver xanthine dehydrogenase and the much studied milk xanthine oxidase (see Bray, 1975 and Coughlan, 1980, for extensive reviews on the properties of these enzymes).

In an attempt to define better the structural differences between the two proteins, which despite having similar molecular weights and identical cofactor composition (one molybdopterin, one FAD, and two Fe₂/S₂ centers/subunit) have very different patterns of reactivity with pyridine nucleotides and molecular oxygen, we decided to employ the strategy of removing the native flavin, FAD, and replacing it with a series of artificial flavins with different redox potentials, as well as with flavins known to be useful as active site probes. The previous paper described the effects of such flavin replacements on catalytic activity, and in some instances, also described the changes in rapid reaction kinetics brought about by the flavin replacements (Nishino et al., 1989b). Similar studies have been carried out in the past with the milk oxidase (Hille et al., 1981; Hille and Massey, 1985) and provided valuable experimental evidence in support of the rapid equilibrium hypothesis of Olson (Olson et al., 1974a) that reducing equivalents taken into the enzyme (at the molybdenum center) from xanthine, are, after dissociation of the product, urate, rapidly distributed among the various redox centers on the basis of their reactive redox potentials. Similarly, it had also been proposed that in the reaction of the reduced enzyme with O₂, it was the reduced flavin which reacted with O₂, and that the other redox centers served largely as electron sinks in rapid equilibrium with the flavin (Olson et al., 1974b). The results which we have obtained with chicken liver xanthine dehydrogenase are also fully consistent with the rapid equilibrium concept, and add further experimental support to it.

One suggestive finding in the previous paper was that 6-OH-FAD binds to the chicken liver enzyme at pH 7.8 as the neutral flavin species (Nishino et al., 1989b). In free solution, 6-OH-FAD has a pK of 7.1 (Mayhew et al., 1974), so it was clear that the pK of the flavin was perturbed on binding to the protein, with stabilization of the neutral flavin species. In a previous study of 6-OH-FAD bound to milk xanthine oxidase (Hille et al., 1981), the spectrum of the bound flavin was that of the anionic form. Since the 6-OH-FAD enzyme was studied only at pH 8.5, the finding of an anionic flavin spectrum was not surprising. However, it did raise the possibility of quite different protein environments around the flavin in the two enzymes. Thus it was of considerable interest

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† To whom correspondence should be addressed.
to study the spectral properties of several ionizable flavins bound to chicken liver xanthine dehydrogenase, and to compare these with the same flavins bound to milk xanthine oxidase. It is found that while the milk oxidase has a slight preference for binding the anionic or benzoquinoid forms of 6-OH-FAD, 6-mercapto-FAD, 8-OH-FAD, and 8-mercapto-FAD, the chicken liver enzyme stabilizes strongly the neutral (protonated) forms of these flavins.

**MATERIALS AND METHODS AND RESULTS AND DISCUSSION**

**CONCLUSIONS**

Milk xanthine oxidase and chicken liver xanthine dehydrogenase share many properties (see Coughlan 1980, for a review). Thus, they have similar molecular weights and each contains the same set of four redox centers, a molybdopterin, two separate Fe-S centers, and FAD. Previous papers in this series have examined the rapid reaction kinetics of the chicken liver enzyme (Schofer et al., 1988), the differences in O₂ reactivity of the two enzymes (Nishino et al., 1989a) and the properties of chicken liver xanthine dehydrogenase substituted with a series of modified flavins of different redox potentials (Nishino et al., 1989b). The present paper describes the properties of both enzymes reconstituted with a series of chemically reactive flavins, or ones which are sensitive to the nature of the protein environment in which the flavin is embedded. The results show dramatic differences between the two enzymes which must reflect the nature of the flavin-protein interactions. In both enzymes the flavin 8-position appears to be exposed to solvent, since 8-mercapto-FAD forms of both react readily with iodoacetamide and MMTS. In the case of milk xanthine oxidase, the flavin 6-position also appears to be quite accessible to solvent, since the 6-SCN-FAD enzyme is converted very rapidly to the 6-mercapto-FAD form with DTT, and since the 6-mercapto-FAD enzyme reacts readily with iodoacetamide, MMTS, H₂O₂, and N-ethylmaleimide, and since the 6-S-S-CH₃-FAD enzyme reacts rapidly with DTT to regenerate the 6-mercapto-FAD enzyme. On the other hand, the flavin 6-position in chicken liver xanthine dehydrogenase appears to be significantly less exposed. The 6-SCN-FAD enzyme does not react with DTT, and the 6-mercapto-FAD enzyme is markedly less reactive with iodoacetamide and the other thiol reagents tested than is 6-mercapto-FAD milk xanthine dehydrogenase, and the 6-S-S-CH₃-FAD enzyme reacts only very slowly with DTT. Since the 8-mercapto-FAD forms of both milk xanthine oxidase and chicken liver xanthine dehydrogenase react readily with iodoacetamide and MMTS, and since the 8-mercapto-FAD enzymes are regenerated rapidly from the 8-S-S-CH₃-FAD forms with DTT, it follows that the different results found for the 6-substituted FAD enzymes are due to different accessibility of the flavin 6-position in milk xanthine oxidase and chicken liver xanthine dehydrogenase, rather than to different reactivities due to stabilization of the neutral flavin in one case (chicken liver xanthine dehydrogenase) and the anion in the other (milk xanthine oxidase).

By far the most dramatic differences between the two enzymes are shown with the FAD derivatives containing an ionizable —OH or —SH residue. While the milk oxidase has either no preference (eg. 8-OH-FAD and possibly 8-mercapto-FAD) or a slight preference for the anionic form (6-OH-FAD and 6-mercapto-FAD), the chicken liver dehydrogenase has a very strong preference for binding the neutral (protonated) forms of all four flavins, with perturbations of the pK by 2-4 pH units. The only other case that we are aware of where the neutral forms of these flavins are stabilized strongly is with hen egg white riboflavin-binding protein (Massey et al., 1979; Massey, 1980; Ghisla et al., 1986; Ghisla and Massey, 1986). While there are several ways in which such stabilization might be achieved, the simplest explanation would be the existence of a negatively charged region in the protein, which prevented binding of the anionic flavin. This would appear the likely explanation with riboflavin-binding protein, which has been found to possess a cluster of negative charges (15/14 residues) in the amine acid sequence 186-199 (White and Merrill, 1988). With all four flavins, the negative charge of the anion can be distributed throughout the whole flavin ring system, with a predominant resonance form having the negative charge in the flavin N(1)-C(2)=O locus (Mayhew et al., 1974; Ghisla and Mayhew, 1976; Massey et al., 1979; Ghisla et al., 1986). As the flavin 8-position is exposed to solvent, the possibility that the enzyme flavin contains the neutral xanthine dehydrogenase, it is unlikely that there can be any negatively charged residue of the protein in this region; it is therefore more likely that the negative charge of the protein is located near the flavin N(1)-position in the dehydrogenase, and that this charge is absent in the oxidase. In the case of 8-mercapto-FAD milk xanthine oxidase, the wave-length maximum of the bound flavin (λmax=579 nm) indicates that it is stabilized as the benzoquinoid anion (Massey et al., 1979) (see Scheme I). Based on the position of this maximum, the extent of stabilization appears to be weaker than with some enzymes where a strong positive charge is thought to be located in the region of the flavin N(1)-position (eg. lactate oxidase, λmax = 607 nm (Massey et al., 1979); d-amino acid oxidase, λmax = 595 nm (Filzpatrick and Massey, 1983)), but is similar to that found for 8-mercapto-FAD p-hydroxybenzoate hydroxylase, where the λmax is at 547 nm in the absence of substrate and 565 nm in the presence of p-hydroxybenzoate (Massey et al., 1979). The stabilization of the anionic flavin forms of p-hydroxybenzoate hydroxylase has been correlated with a partial positive charge from the N-terminal portion of an α-helix of the protein directed toward the flavin N(1)-position (Hofsteenge et al., 1980) and suggests a better orientation of this helix toward the flavin N(1)-position in the enzyme-substrate complex than in substrate-free enzyme. The present results with 8-mercapto-FAD milk xanthine oxidase suggest a similar interaction.

Differences in structure of chicken liver xanthine dehydrogenase and milk xanthine oxidase in the reduced state with the native FAD-containing enzymes is also evident from their different reactivities with iodoacetamide. Xanthine-reduced milk xanthine oxidase has been shown to react with iodoacetamide, resulting in alkylation of the reduced flavin at the C(4a)-position, yielding enzyme containing the catalytically inactive C(4a)-acetamido FAD (Komai and Massey, 1971). In contrast, chicken liver xanthine dehydrogenase reacted under the same conditions as those giving rapid inactivation with milk xanthine oxidase (0.1 M phosphate, pH 6.3, 1 mM xanthine or excess Na₂S₂O₄, 1 mM iodoacetamide, 25 °C, under anaerobiosis) gave only marginal loss of activity over 70 min and no indication of alkylation of the enzyme flavin, as judged by the return of the original absorption spectrum on reoxidation. These results clearly imply that the flavin C(4a)-

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1 Portions of this paper (including “Materials and Methods,” “Results and Discussion,” Figs. 1-11, Tables I-III, and Scheme I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: MMTS, methylmethanethiosulfonate; XDH, chicken liver xanthine dehydrogenase; MXX, milk xanthine oxidase; DTT, dithiothreitol.

3 T. Nishino and V. Massey, unpublished results.
position in substrate-reduced milk xanthine oxidase is considerably more accessible than in reduced chicken liver xanthine dehydrogenase (substrate-reduced or chemically-reduced).

The results with modified flavin forms of milk xanthine oxidase and chicken liver xanthine dehydrogenase would predict that in the reversible interconversion of xanthine dehydrogenase and xanthine oxidase, which can occur, for example, with the enzyme from rat liver (Stirpe and Della Corte, 1969; Waud and Rajagopalan, 1976a, 1976b) by oxidation and reduction of suitably positioned thiol residues (Della Corte and Stirpe, 1972; Waud and Rajagopalan, 1976b; Saito and Nishino, 1989), substantial changes in protein conformation must occur. Thus, the dehydrogenase form, with intact NAD-binding site, and a strong negative charge located near the flavin N(1)-position, would change its conformation on oxidation of the crucial thiol residues in such a manner that the NAD-binding site was lost at the same time as the negatively charged protein residue was removed from the vicinity of the flavin N(1)-position, and replaced instead by a partial positive charge, such as could be provided from amide nitrogens or the C-term region of an α-helix. Evidence that such conformational changes may indeed occur in a reversible fashion with rat liver xanthine dehydrogenase has been obtained in a similar study using the same flavin derivatives as reported here, and will be described in full in a future publication.

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REFERENCES

Barber, M. J., Bray, R. C., Lowe, D. J., and Coughlan, M. P. (1976) Biochem. J. 153, 297–307
Barber, M. J., Coughlan, M. P., Kanda, M., and Rajagopalan, K. V. (1980) Arch. Biochem. Biophys. 201, 468–475
Bray, R. C. (1975) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol. XII, pp. 300–420, Academic Press, Orlando, FL
Cleere, W. F., and Coughlan, M. P. (1975) Comp. Biochem. Physiol. 50B, 311–322
Coughlan, M. P. (1986) in Molybdenum and Molybdenum-containing Enzymes (Coughlan, M. P., ed) pp. 121–185, Pergamon Press, Oxford
Della Corte, E., and Stirpe, F. (1972) Biochem. J. 126, 739–745
Fitzpatrick, P. F., and Massey, V. (1983) J. Biol. Chem. 258, 9700–9705
Ghisla, S., and Mayhew, S. G. (1980) Methods Enzymol. 66, 241–253
Ghisla, S., and Massey, V. (1986) Biochem. J. 239, 1–12
Ghisla, S., Massey, V., and Yagi, K. (1986) Biochemistry 25, 3282–3289
Hille, R. (1980) in The Chemistry and Uses of Molybdenum (Barry, H. F., and Mitchell, P. C. H., eds) pp. 310–315, Clarinex Molybdenum Co., Ann Arbor, MI
Hille, R., and Massey, V. (1985) in Molybdenum Enzymes (Spiro, T. G., ed) Vol. 7, pp. 443–518, Wiley-Interscience Publishing, New York

Hille, R., Fee, J. A., and Massey, V. (1981) J. Biol. Chem. 256, 8903–8940
Hofsteeiene, J., Verijken, J. M., Weijer, W. J., Beintema, J. J., Wergova, R. K., and Drerth, J. (1980) Eur. J. Biochem. 113, 141–150
Komai, H., and Massey, V. (1971) in Flavins and Flavoproteins (Kamin, H., ed) pp. 399–415, University Park Press, Baltimore
Komai, H., Massey, V., and Palmer, G. (1969) J. Biol. Chem. 244, 1692–1700
Krauth-Siegel, R. L., Schirmer, R. H., and Ghisla, S. (1985) Eur. J. Biochem. 148, 335–344
Lowe, H. J., and Clarke, W. M. (1956) J. Biol. Chem. 221, 983–992
Massey, V., and Hemmerich, P. (1976) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol. XII, pp. 191–212, Academic Press, New York
Massey, V., and Hemmerich, P. (1978) Biochemistry 17, 9–17
Massey, V. (1980) in Photochemistry and Sensory Transduction in Aequor Organisms (Lenci, F., and Colombetti, G., eds) pp. 255–269, Plenum Press, New York
Massey, V., Brumby, P. E., Komai, H., and Palmer, G. (1969) J. Biol. Chem. 244, 1682–1691
Massey, V., Komai, H., Palmer, G., and Eison, G. B. (1970) J. Biol. Chem. 245, 2837–2844
Massey, V., Ghisla, S., and Moore, E. G. (1979) J. Biol. Chem. 254, 9640–9650
Massey, V., Claiborne, A., Biemann, M., and Ghisla, S. (1984) J. Biol. Chem. 259, 9667–9678
Massey, V., Ghisla, S., and Yagi, K. (1986) Biochemistry 25, 8103–8112
Mayhew, S. G., Whitfield, C. D., and Ghisla, S. (1974) Eur. J. Biochem. 44, 579–591
Moore, E. G., Cardemil, E., and Massey, V. (1978) J. Biol. Chem. 253, 633–642
Moore, E. G., Ghisla, S., and Massey, V. (1979) J. Biol. Chem. 254, 8173–8178
Nishino, T. (1974) Biochem. Biophys. Acts 341, 93–98
Nishino, T., Nishino, T., Schofer, L. M., and Massey, V. (1989a) J. Biol. Chem. 264, 2518–2527
Nishino, T., Nishino, T., Schofer, L. M., and Massey, V. (1989b) J. Biol. Chem. 264, 6075–6085
Olson, J. S., Baiou, D. B., Palmer, G., and Massey, V. (1974a) J. Biol. Chem. 249, 4350–4362
Olson, J. S., Baiou, D. B., Palmer, G., and Massey, V. (1974b) J. Biol. Chem. 249, 4363–4392
Palmer, G., and Olson, J. S. (1980) in Molybdenum and Molybdenum-containing Enzymes (Coughlan, M. P., ed) pp. 189–220, Pergamon Press, Oxford
Rajagopalan, K. V., and Handler, P. (1967) J. Biol. Chem. 242, 4097–4107
Saito, T., and Nishino, T. (1989) J. Biol. Chem. 264, 10015–10022
Schofer, L. M., Massey, V., and Claiborne, A. (1981) J. Biol. Chem. 256, 7329–7337
Schofer, L. M., Massey, V., and Nishino, T. (1988) J. Biol. Chem. 263, 13528–13538
Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959) Arch. Biochem. Biophys. 82, 288–299
Spencer, R., Fisher, J., and Walsh, C. (1976) Biochemistry 15, 1043–1053
Steenkamp, D. J., McIntire, W., and Kenney, W. C. (1978) J. Biol. Chem. 253, 2818–2824
Stirpe, F., and Della Corte, E. (1969) J. Biol. Chem. 244, 3855–3863
White, H. B., and Merrill, A. H. (1988) Ann. Rev. Nutr. 8, 279–299
Waud, W. R., and Rajagopalan, K. V. (1976a) Arch. Biochem. Biophys. 172, 354–364
Waud, W. R., and Rajagopalan, K. V. (1976b) Arch. Biochem. Biophys. 172, 365–379
Williams, C. H., Jr., Ansnett, L. D., Matthews, R. G., Thorpe, C., and Wilkinson, K. D. (1979) Methods Enzymol. 62, 185–198

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RESULTS AND DISCUSSION

6-Hydroxy-FAD MXO; comparison with 6-OH-FAD XDH

Figure 1 shows the absorption spectrum of deflavoenzyme reconstituted with 6-OH-FAD. The spectrum of the starting flavin is not apparent; the absorption bands of the enzyme-bound 6-OH-FAD are broadened and the intensity is decreased. The absorption spectrum was recorded with an Aminco spectrophotometer.

6-Mercapto-FAD XDH

In results analogous to those found with 6-OH-FAD, deflavo-XDH binds 6-mercaptopo-FAD tightly as the neutral flavin. Figure 2 shows the difference spectrum obtained at pH 7.8 between deflavoenzyme reconstituted with 6-mercaptopo-FAD and the native deflavo-FAD XDH. The spectrum of the flavin was calculated on the basis of the flavin content determined by the absorption spectrum of the deflavo-FAD XDH (Nishino et al., 1989b). The difference spectrum of the reconstituted enzyme is shown in Figure 2. The major changes are the broadening of the bands of the flavin and a decrease in the intensity of the absorption bands. The difference spectrum is in agreement with the results obtained with 6-OH-FAD.

The flavin 6-position appears to be accessible to solventborne reagents, since the 6-mercaptopo-FAD enzyme reacts rapidly with methyleneblue and with hemoglobin (Hb), to yield the flavin 6-S-S-S-CH$_3$ derivative, with the spectral changes shown in Figure 3. The 6-mercaptopo-FAD enzyme is likewise very sensitive to reaction with excess diethylthiocarbamate, indicating that this reaction may have taken place in all cases. In addition, the 6-mercaptopo-FAD enzyme gives an almost complete disappearance of the
flavin chromophores (Figure 3) (Steinmetz et al., 1978). The difference spectrum obtained on reaction with H-mercaptoethanol agent confirms that the 6-mercaptopentyl-FAD is bound as the neutral flavin. While both FAD and NEM react rapidly with the enzyme-bound 6-mercaptopentyl-FAD, iodoacetamide fails to react, even when 10 mM iodoacetamide is incubated for 30 min with the enzyme at pH 7.4, 4°C. Under the same conditions, the reaction of iodoacetamide with free 6-mercaptopentyl-FAD would be complete within seconds (Steinmetz et al., 1980).

The 6-mercaptopentyl-FAD XDH has 23% the xanthine-FAD reductase activity of native enzyme under standard assay conditions, and 12% of the xanthine-G reductase activity (Besenius et al., 1986). As expected from these results, the modified enzyme is readily reduced by xanthine under anaerobic conditions. Figure 3 shows selected spectra obtained on anaerobic titration of the enzyme with xanthine, and the spectrum of the fully reduced enzyme obtained by the addition of dithionite. Distinctively different spectral changes were obtained at different stages of the reduction, as shown in the inset to Figure 4. The first 0.85 mol xanthine per mol enzyme monomer gives a difference spectrum (dashed line, inset) typical of reduced iron-sulfur centers (Sleerkamp et al., 1986). Between 0.5 and 1.75 mol xanthine, the difference spectrum (bottom solid line, inset) can be attributed almost entirely to the reduction of the 6-mercaptopentyl-FAD, while the difference spectrum between 1.7-3 mol xanthine contains contributions both from the flavin and the second iron-sulfur center. These results clearly indicate that one of the Fe/S centers has the highest redox potential (by analogy, with native enzyme this should be Fe/S II (Bartner et al., 1986). Schopfer et al., 1986, followed by 6-mercaptopentyl-FAD, and with Fe/S I having the lowest potential of the chromophores absorbing in the visible wavelength region.

Figure 3. Reactivity of 6-mercaptopentyl-FAD XDH. Top solid line: 6-mercaptopentyl-FAD XDH in 0.05 M phosphate buffer, pH 7.4 plus 0.2 mM EDTA, 4°C. Top dashed line: 1 min after reaction with 8.1 mM methylmethanethiosulphonate (MMTS). Bottom dashed line: separate experiment, 1 min after reaction with 8.1 mM H-ethylisothiouronium (HEIT). Lower solid line: difference spectrum of reaction with NEM. 6-Mercaptopentyl-FAD XDH.

Figure 5 shows the spectrum of deflavoenzyme before and after reconstitution with 6-mercaptopentyl-FAD, and by difference, the contribution of the enzyme-bound flavin (see also Table I). In distinction to the results with XDH, it is clear from the long wavelength band that the flavin is present in the enzyme in its anionic form. The spectrum is not altered by lowering the pH to 5.5, which is the pH of the 6-mercaptopentyl-FAD free in solution (Ghisla et al., 1986). Thus we can estimate an upper limit for the pH of the enzyme-bound flavin as pH 5.2, again indicating some preference of the metal oxides for binding the anionic flavin, as opposed to the strong preference of the chicken liver dehydrogenase to bind the neutral species. 6-Mercaptopentyl-FAD XDH, like the analogous chicken liver dehydrogenase, reacts rapidly with MTS and NEM. In this case, consistent with the 6-mercaptopentyl-FAD being bound as the anion, the spectral changes accompanying the reaction result in a complete loss of the absorbance in the 500-600 nm region associated with the 6-mercaptopentyl-FAD (see next section, Figure 6). Both modifications are reversed by the subsequent addition of DTT, with complete restoration of the initial absorption spectrum. 6-Mercaptopentyl-FAD XDH also reacts rapidly with H2O2 to yield presumably the 6-SCH-FAD derivative, since the resulting spectrum is similar to that of native enzyme, and the reaction is not reversed by incubation with DTT (results not shown). These properties are similar to those found previously for 6-mercaptopentyl-FAD oxidized beyond the sulfenic oxidation level (Steinmetz et al., 1983). All enzyme forms are catalytically active; the activities will be documented in a later section. These results indicate that with XDH the flavin reaction is accessible to oxidants. However, it would appear that the accessibility in the case of XDH is appreciably greater than with XDH, since the reoxidation of 6-mercaptopentyl-FAD XDH following reaction with DTT is virtually instantaneous with PMS, but quite slow with XDH (see previous section). Also, while no reaction of 6-mercaptopentyl-FAD XDH with iodoacetamide was detected, 6-mercaptopentyl-FAD XDH reacted reasonably fast (300-600 nm absorbance) at pH 6.5, 4°C. (results not shown).

6-SCN-FAD XDH and 6-SCN-FAD XDH.

Differences in the active site structure of the chicken liver dehydrogenase and the milk oxidase are also apparent from the very different properties of the two enzymes.

Figure 5. Spectral properties of 6-mercaptopentyl-FAD XDH. Dashed line: deflavoenzyme. Top solid line: reconstituted enzyme. Lower solid line: spectral contribution of the enzyme bound 6-mercaptopentyl-FAD. Conditions: 0.1 M Tris buffer, pH 8.0 plus 0.5 mM EDTA, 4°C.

Figure 6. Reactivity of 6-SCN-FAD XDH. Dashed line: + 0.1 M HgSCN. Top solid line: 6-SCN-FAD XDH in 0.1 M phosphate buffer, pH 7.4 plus 0.2 mM EDTA, 4°C. Top solid line: 1 min after addition of 0.22 mM DTT. Curve 2: 1 min after addition of 0.22 mM DTT. Curve 3: 1 min after additional addition of 0.5 mM NEM. Inset: Spectra of conversion of 6-SCN-FAD to 6-SCN-FAD XDH by DTT under the above conditions, measured by following the increase in A450. Dashed circles, free 6-SCN-FAD.
dehydrogenase is that occurring with β-mercapto-FAD. This is illustrated in Figure 7, which shows the spectra of the two separate components, deflavo-XDH and β-mercapto-FAD. On mixing, the 535 nm absorbance of free β-mercapto-FAD disappears rapidly (less than 1/2 wave at 47°C) so that the final absorbance in the 500 nm region is only slightly greater than that of the initial deflavo-XDH. The difference spectrum between reconstituted and deflavo-XDH has a sharp peak at 529 nm (see Table I) and has the characteristics of neutral (protonated) B-5-enaza-5-deoxyflavin (Poore et al., 1979). The pit of the free flavin (in 1.9 M NaCl, Poore et al., 1979) is shifted by 0.8 nm in favor of the flavin being in a fully protonated state, the pK 2.3-2.5 shift of 2.45 nm units.

On anaerobic titration with xanthine, Figure 8 shows a sharp bleaching of both the flavin and FeS/F7. Chromophores, with a simultaneous accumulation of covalent semiquinone, as judged by the increased absorbance in the 600 nm region which disappears only on the addition of dimethylsulfoxide (Figure 8, inset). The simultaneous loss in ASSD and increase in A540 are caused by the redox potential of the highest potential center, Fe/F7, being close to that of the 8-mercapto-FAD/8-mercapto-FADD couple. The fact that the increased absorbance at 590 nm persists until the addition of dimethylsulfoxide suggests that the potential of the 8-mercapto-FAD/8-mercapto-FADD couple is quite low, possibly lower even than that of the low potential iron center Fe/F5.1. No significant contribution of the Fe/F7 contribution to the spectrum could be observed.

β-mercapto-FAD-XDH

In keeping with the anionic nature of the bound flavin, the redox potential of the flavin in β-mercapto-FAD might be expected to be similar to that of native enzyme-bound 8-mercapto-FAD, a contribution of 8-mercapto-FAD to xanthine dehydrogenase. If preformed 8-mercapto-FAD is added to the enzyme bound 8-mercapto-FAD, the spectrum changes reversibly in the anionic form. With the protein-flavin interaction stabilizing the semiquinone, as judged by the decrease in absorbance at 579 nm in the anionic form. With the protein-flavin interaction stabilizing the semiquinone, as judged by the decrease in absorbance at 579 nm in the anionic form.

β-mercapto-FAD-XDH

In keeping with the pattern shown with the other flavins already described, 8-CH2-FAD was found to bind to deflavo-XDH in the neutral (protonated) state (Figure 10 and Table I). The spectral characteristics of the bound flavin (λmax 444 nm, ε292 2.17 mmole”1 cm”1) are typical of those of the neutral flavin (Ghisla and Mayhew, 1976). In free solution, 8-CH2-FAD has a pit of 3.5, when bound to XDH the pit is ε5.5. Thus, as in the case of 8-mercapto-FAD, there is a remarkable stabilization of the neutral form of the flavin with the pit being perturbed by at least 1.5 pH units.

As described in the previous paper (Nishino et al., 1980), the 8-CH2-FAD enzyme has xanthine/O2 reductase activity which is ε50% that of native enzyme but very low xanthine-O2 reductase activity (1% of that of native enzyme). In keeping with this, the aerobic addition of semiquinone results in a partially reduced spectrum which stage at a constant steady state level until the xanthine is exhausted. There was no obvious presence of flavin semiquinone in such experiments; the difference spectrum between the oxidized enzyme and aerobic steady state can be accounted for by reduction of 12 FeS/chromophores and approximately 30% reduction of the bound flavin (results not shown). The neutral semiquinone form of 8-CH2-FAD has a high extinction at 580 nm (ε444 2.17 mmole”1 cm”1) and so should have been detectable resin it had not been a significant contributor to the steady state spectrum. Hence, we can conclude, as we have done in the previous papers (Nishino et al., 1980), that the flavin bound to xanthine dehydrogenase.

β-mercapto-FAD-MXO

The spectral characteristics of 8-CH2-FAD bound to xanthine oxidase are shown in Figure 10 and are in every respect identical to those of the flavin bound to xanthine dehydrogenase. The spectral characteristics of the bound flavin at pH 7 is characterized by two peaks of almost equal intensity at 475 and 406 nm, with extinction coefficients ε475 3.1 mmole”1 cm”1. This is typical of 8-CH2-FAD in the anionic state (Ghisla and Mayhew, 1976). The absorption spectrum of the reconstituted complex is unchanged on lowering the pH to 5.4, below which the spectrum changes reversibly in the pattern expected for protonation of the 8-CH2-FAD flavin.

Figure 8. Anaerobic titration of β-mercapto-FAD XDH with xanthine. Curve a: 2.4 pH 6.8 β-mercapto-FAD XDH in 0.05 M phosphate buffer, pH 7.8, 0.1 M EDTA, 4°C, before addition of xanthine. Curve b: 2.5 pH 6.8, after addition of 3.35, 11, 15.6, 15.6 μM xanthine per mg enzyme-bound flavin. Curve c: after addition of dimethylsulfoxide. Percent ΔA456 and ΔA490 as a function of semiquinone per flavin molecule in the oxidized state. The change between oxidized enzyme and reduction with dimethylsulfoxide are also shown during the titration.
Protein Structural Differences between XDH and M XO

Figure 10. Spectral properties of 8-hydroxy-FAD XDH. Dashed line: deflavoenzyme. Solid line: enzyme reconstituted with B-Oh-FAD. Lower solid line: spectral contribution of enzyme-bound B-Oh-FAD. Conditions: 0.05 M phosphate buffer, pH 7.0 plus 0.2 mM EDTA.

Figure II. Spectral properties of 8-hydroxy-FAD M XO. Dashed line: deflavoenzyme. Solid line: enzyme reconstituted with B-Oh-FAD. Lower solid line: spectral contribution of enzyme-bound B-Oh-FAD. Conditions: 0.05 M phosphate buffer, pH 7.0 plus 0.2 mM EDTA.

Table II

| Engine form | Vmax (mmol/min) | Km(xanthine) (µM) | Km(NAD) (µM) |
|-------------|----------------|-------------------|--------------|
| Native XDH  | 1600           | 20                | 24           |
| B-Oh-FAD XDH| 1200           | 2                 | 87           |
| 6-mercaptopo-FAD XDH | 330       | 4                  | 7            |
| 6-mercaptopo-FAD M XO | 880       | 14                 | 10           |

a) Initial velocities of the xanthine/NaNO3 reductase activity were measured at 25°C in 0.05 M potassium phosphate buffer pH 7.8 containing 0.2 mM EDTA. Xanthine concentrations were varied from 10-56 µM and NAD concentrations from 10-500 µM. The production of NaNO3 was monitored at 340 nm.

Table III

| Engine reconstituted with | Activity relative to native enzyme (%) | Ent pH 7 free flavin (µM) |
|---------------------------|----------------------------------------|--------------------------|
| FAD                       |                                        |                          |
| e-SCN-FAD                 | 60 ± 14                                | -29 ± 5                  |
| e-6-mercaptopo-FAD        | 40                                     | 70                       |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |
| 6-mercaptopo-FAD oxalate  | not determined                         |                          |

a) Assay were carried out at 25°C in air saturated 0.1 M potassium phosphate buffer, pH 8.5, containing 0.15 M xanthine, and following the production of UMP 1 mg/ml. The activities are reported as a percentage of that obtained with native enzyme on an enzymic basis, using the extinction coefficients for the reconstituted enzymes, reported in Table I. Lowe and Clarke, 1956.

Table I

| Spectral Contribution | E-S-S-CH3-FAO k, | 6-mercapto-FAD | 6-OH-FAD | 6-mercapto-FAD 4-thio-FAD | 6-mercapto-FAD 3-thio-FAD | 6-mercapto-FAD 5-thio-FAD | 6-mercapto-FAD 6-thio-FAD |
|-----------------------|-----------------|----------------|-----------|---------------------------|---------------------------|---------------------------|---------------------------|
| Native XDH            | 100             | 65             | 95         | 100                       | 95                       | 100                       | 95                        |

b) Values reported are initial rates. The activity decreases rapidly during the assay due to conversion of the flavin to an unidentified product.

c) The reaction product of 6-mercaptopo-FAD oxalate with PMS (leading to 12T). The reaction product of 6-mercapto-FAD oxalate with PMS (leading to 12T). The reaction product of 6-mercapto-FAD oxalate with PMS (leading to 12T). The reaction product of 6-mercapto-FAD oxalate with PMS (leading to 12T).