Differences in Environment of FAD between NAD-dependent and O$_2$-dependent Types of Rat Liver Xanthine Dehydrogenase Shown by Active Site Probe Study*

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Rat liver deflavoxanthine dehydrogenase has been prepared by incubating native enzyme with calcium chloride. On reconstitution with FAD, about 85% of the original activity is recovered, all which is the O$_2$-dependent type. In contrast, when dithiothreitol-treated deflavoenzyme is incubated with FAD, the recovery of activity is almost the same as above, but most of the recovered activity is of the NAD-dependent type. Deflavoenzyme with or without previous treatment with dithiothreitol was also reconstituted with two artificial FAD analogues, 8-mercapto-FAD and 6-OH-FAD. The difference spectra between the reconstituted enzymes and the initial deflavoenzyme indicate that, in each case, the FAD analogue is bound in its neutral form in dithiothreitol-treated enzyme, whereas it is bound in the anionic form in enzyme without previous dithiothreitol treatment. Furthermore, the protonated forms can be converted into the anionic forms on storage with a concomitant change of activity from the NAD-dependent to the O$_2$-dependent type. This clearly indicates different environments around FAD in the two types of enzyme protein, which are shown to be interconvertible through oxidation-reduction of enzyme cysteinyl residues.

Mammalian xanthine dehydrogenases exist as the NAD-dependent type (type D) in freshly prepared samples, i.e. they exhibit low xanthine:O$_2$ reductase activity but high xanthine:NAD reductase activity even in the presence of O$_2$ (Della Corte and Stirpe, 1968, 1972; Stirpe and Della Corte, 1969; Nakamura and Yamazaki, 1982). However, during purification, most of the enzymes are converted to the O$_2$-dependent type (type O), i.e. they exhibit low xanthine:NAD reductase activity but high xanthine:O$_2$ reductase activity even in the presence of NAD (Della Corte and Stirpe, 1968, 1972; Stirpe and Della Corte, 1969; Nakamura and Yamazaki, 1982). This conversion occurs reversibly through oxidation of sulfhydryl groups or irreversibly through proteolysis (Della Corte and Stirpe, 1972; Stirpe and Della Corte, 1969; Waud and Rajagopalan, 1976b; Nakamura and Yamazaki, 1982). Although the enzyme can be easily converted to the irreversible O$_2$-dependent type during purification, it can be purified as a reversibly interconvertible type by careful purification procedures (Waud and Rajagopalan, 1976a; Nakamura and Yamazaki, 1982; Ikegami and Nishino, 1986). The enzyme consists of two identical subunits; and each subunit contains one FAD, two iron-sulfur centers, and one molybdopterin as cofactors (Bray, 1975).

Using the reversibly interconvertible rat liver enzyme, it has been shown recently that, in reductive titrations, the type D enzyme can form a stable FAD neutral semiquinone, but the type O enzyme cannot (Saito and Nishino, 1989). The type D enzyme also shows appreciable semiquinone during turnover with xanthine and oxygen as substrates, but the type O enzyme does not (Waud and Rajagopalan, 1976b; Saito and Nishino, 1989). Furthermore, the $K_m$ for oxygen of the type D enzyme is about five times as high as that of the type O enzyme (Saito and Nishino, 1989). These differences between the two types of rat liver enzyme are quite similar to those between chicken liver xanthine dehydrogenase, which has never been shown to be convertible to the O$_2$-dependent type, and milk xanthine oxidase, which is usually isolated as the O$_2$-dependent type (Schopfer et al., 1988; Nishino et al., 1989a; Massey et al., 1969; Olson et al., 1974). These observations suggest that the protein environment of enzyme-bound FAD is different between the rat liver type D and O enzymes in a similar way as it is between the chicken liver dehydrogenase and the milk oxidase.

In a preceding paper (Massey et al., 1989), deflavoenzymes prepared from milk xanthine oxidase and chicken liver xanthine dehydrogenase were reconstituted with several artificial flavins, which have been proved to be very useful as active site probes. FAD analogues containing ionizable —OH or —SH substituents at the flavin 6- or 8- position can exist in forms with their substituents protonated (neutral) or deprotonated (anionic) (Moore et al., 1979; Mayhew et al., 1974). Each structure has a characteristic absorption spectrum (Moore et al., 1979; Mayhew et al., 1974), so it can be easily determined which form of the flavin is stabilized in the particular protein. In preceding papers (Hille et al., 1981; Massey et al., 1989; Nishino et al., 1989b), it has been clearly shown that these artificial flavins take the anionic form in the milk oxidase and the neutral form in the chicken liver dehydrogenase. This fact indicates that the environment of...
FAD is different in the chicken liver dehydrogenase and the bovine milk oxidase, predicting that such a change of the environment of flavin may occur in the reversible interconversion between the rat liver type D and O enzymes. However, it is also possible that such differences in FAD environment are not directly related to the difference in catalytic function of the two enzymes, but are merely due to different proteins from different species.

In this paper, we demonstrate that differences in the environments of FAD, quite similar to those observed between the chicken liver dehydrogenase and the milk oxidase, are indeed present between the two types of rat liver enzyme and that the two protein structures seemed to be interconvertible through oxidation-reduction of cysteiny1 residues.

MATERIALS AND METHODS

Artificial FAD analogues were prepared as reported previously: 6-OH-FAD (Krauth-Siegel et al., 1985) and 8-mercapto-FAD (Moore et al., 1979). Calcium chloride was from Merck (suprapure grade).

Enzyme was purified from rat liver by a previously reported method (Ikegami and Nishino, 1986). The concentration of native enzyme was determined using \( E_{450} = 35,800 \, \text{M}^{-1} \, \text{cm}^{-1} \) as the molar extinction coefficient of the enzyme subunit (Johnson et al., 1974). The subunit concentration of deflavoenzyme was determined from the absorbance at 550 nm using \( E_{550} = 10,900 \, \text{M}^{-1} \, \text{cm}^{-1} \). Concentrations of enzymes reconstituted from DTT-untreated and -treated deflavoenzymes with 8-mercapto-FAD were determined using the experimentally determined values of \( E_{450} = 41,200 \, \text{M}^{-1} \, \text{cm}^{-1} \) and \( E_{550} = 38,200 \, \text{M}^{-1} \, \text{cm}^{-1} \), respectively. Concentrations of enzymes reconstituted from DTT-untreated and -treated deflavoenzymes with 6-OH-FAD were determined using the experimentally determined values of \( E_{450} = 42,500 \, \text{M}^{-1} \, \text{cm}^{-1} \) and \( E_{550} = 43,900 \, \text{M}^{-1} \, \text{cm}^{-1} \).

Xanthine:NAD\(^+\) and xanthine:O\(_2\) oxidoreductase activities were assayed at 25 °C with or without 0.5 mM NAD in a 3-ml reaction mixture containing 50 mM potassium phosphate buffer, pH 7.8, 0.2 mM EDTA, and 0.15 mM xanthine (Ikegami and Nishino, 1986). Xanthine:NAD oxidoreductase activity (NAD-dependent activity) was determined by following the absorbance of NADH at 340 nm. Xanthine:O\(_2\)-oxidoreductase activity (O\(_2\)-dependent activity) was determined by following the absorbance change at 295 nm due to the formation of uric acid. The activity-to-flavin ratio was obtained by dividing the change of absorbance per minute at 295 nm in the presence of NAD under aerobic conditions by the absorbance at 450 nm of the enzyme used in the assay. Xanthine:di chloroindophenol oxidoreductase activity was assayed in a 3-m1 reaction mixture containing 50 \( \mu \)M 2,6-dichloroindophenol, 150 \( \mu \)M xanthine, 50 mM potassium phosphate buffer, pH 7.8, and 0.2 mM EDTA following the decrease in absorbance at 600 nm to 25 °C.

Preparation of Rat Liver Deflavoxanthine Dehydrogenase—Rat liver deflavoxanthine dehydrogenase was prepared according to a previously reported method (Komai et al., 1969) with minor modifications. Ice-cold 4.0 M calcium chloride solution was added to an equal volume of freshly purified ice-cold rat liver xanthine dehydrogenase in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.4 mM EDTA and 1 mM sodium salicylate. The calcium chloride solution was added little by little while the mixture was shaken well on ice. Then, the mixture was incubated at 20 °C for 90 min, followed by passage through a small column of Sephadex G-25 to separate deflavoxanthine oxidase from calcium chloride and released flavin. The elution buffer was 0.1 M Tris-HCl buffer, pH 8.0, containing 0.4 mM EDTA. Reconstitution of Deflavoenzyme with FAD—2.3 \( \mu \)M deflavoenzyme was incubated with equimolar FAD in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.4 mM EDTA and 1 mM sodium salicylate at 4 °C in a fluorometric cuvette. The fluorescence emission at 526 nm with excitation wavelength at 450 nm was followed. At the same time, samples were withdrawn periodically from the mixture, and the enzyme activity was determined. After the activity reached a constant level, 1.1 \( \mu \)M FAD was added to check that no further increase in activity occurred. In a parallel experiment, deflavoenzyme was treated with 5.0 mM dithiothreitol at 25 °C for 2 h, followed by incubation with FAD as described above.

Reconstitution of Deflavoenzyme with Artificial FAD Analogues—Both DTT-treated and -untreated deflavoenzymes obtained in the same preparation were reconstituted with each FAD analogue. Deflavoenzyme in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.4 mM EDTA was put in a cuvette, and a substoichiometric amount (0.6-0.75 mol of flavin/mol of deflavoenzyme) of FAD analogue was added. The cuvette was placed in a Hitachi U-3200 spectrophotometer equipped with a temperature-controlled cell unit, and the absorbance spectrum was recorded periodically. The incubation temperature was 10 °C. When the spectral changes associated with flavin binding had ceased, the spectrum of the initial deflavoenzyme was subtracted from the final one, and the spectral contribution of enzyme-bound flavin was calculated. The FAD analogue was further added to make its concentration in slight excess (1.4-1.8 mol of flavin/mol of deflavoenzyme). When the absorbance spectrum became constant, the mixture was incubated at room temperature for an additional 30 or 30 min to attain complete equilibrium, and the spectrum was again recorded. Then, the mixture was concentrated by centrifugation with an Amicon Centriog 30, and the spectrum of the filtrate was recorded. The spectrum of the reconstituted enzyme was calculated by subtraction of the spectrum of the filtrate from that of the mixture measured immediately before concentration. The extinction coefficients of the artificial enzymes were calculated based on the spectrum of the starting deflavoenzyme. In all cases, the difference spectra of enzyme-bound flavin obtained in the two halves of the reconstruction agreed well with each other. Concentration of the reconstituted enzyme by Centrifugation, followed by dilution with buffer, was repeated several times until the spectrum of the filtrate showed no flavin absorbance. The absorbance spectrum of the isolated enzyme was recorded, and its NAD-dependent and O\(_2\)-dependent activities were assayed. In a parallel experiment, deflavoenzyme in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.4 mM EDTA was incubated with 5 mM DTT at room temperature for 2 h, followed by reconstitution with FAD analogues as described above.

RESULTS

Preparation of Deflavoenzyme—Fig. 1 shows the absorbance spectra of rat liver deflavoxanthine dehydrogenase and native enzyme. The former has a broad absorption band extending from 300 to 700 nm, which is characteristic of non-HEME iron and is very similar to those of milk deflavoxanthine oxidase (Komai et al., 1969) and chicken liver deflavoxanthine dehydrogenase (Nishino et al., 1989a). The spectra of deflavoxanthine and native enzyme are almost identical in the wavelength range beyond 550 nm. The difference spectrum between deflavoxanthine and native enzyme yields the spectral contribution of enzyme-bound FAD. As the ratio of absorbance at 450 nm to that at 550 nm is 3.27 in native rat liver xanthine dehydrogenase, the molar extinction coefficients of deflavoxanthine at 550 and 466 nm are calculated to be 10,900 and 22,300 \( \text{M}^{-1} \text{cm}^{-1} \), respectively. The latter is a little lower than those of milk xanthine oxidase and chicken liver xanthine dehydrogenase: \( E_{450} = 25,300 \) and \( 25,200 \, \text{M}^{-1} \, \text{cm}^{-1} \), respectively (Komai et al., 1969; Nishino et al., 1989a). It is possible

FIG. 1. Absorbance spectrum of rat liver deflavoxanthine dehydrogenase. Rat liver deflavoxanthine dehydrogenase was prepared as described under “Materials and Methods.” ---, spectrum of deflavoenzyme; ---, spectrum of native rat liver xanthine dehydrogenase; ----, difference spectrum of the above two.
that a small amount of damaged deflavoenzyme is present in the deflavoenzyme preparations as the recovery of activity was about 85% of that of the original upon reconstitution with FAD (see below). The xanthine-dichloroindophenol oxidoreductase activity of deflavoenzyme was 96% of that of the original enzyme, suggesting that treatment with calcium chloride had little effect on the xanthine-binding site. In most cases, deflavoenzyme had slight xanthine oxidizing activity measured at 295 nm aerobically in the presence of NAD⁺, about 2% of that of the original enzyme.

Reconstitution of Deflavoenzyme with FAD and Effect of Dithiothreitol Treatment of Reconstituted Enzyme and Deflavoenzyme—The time course of increase in enzyme activity measured aerobically at 295 nm in the presence NAD⁺ and that of the decrease in fluorescence emission due to free flavin were almost parallel when equimolar deflavoenzyme and FAD were incubated together (data not shown). The final recovery of activity was 84% of that of the original, after slight excess of FAD (1.5 mol of FAD/mol of deflavoenzyme, final) was further added. The reconstituted enzyme showed no NAD-dependent activity, indicating that all of the reconstituted enzyme was type 0 (Table I, Condition 1). The reconstituted enzyme was then incubated with 5 mM dithiothreitol at 25 °C for 2 h. This incubation resulted in the appearance of NAD dependant activity, and the conversion from the type 0 to D enzyme was estimated to be 60–70% (Table I, Condition 2). After removing excess DTT by repeated centrifugation through a Centricon 30 filter and dilution with buffer, this enzyme was incubated with 1 mM 4,4'-dithiodipyridine at room temperature for 70 min. This incubation resulted in a 90% decreased in NAD-dependent activity and concomitant increase in O₂-dependent activity. Thus, reconstituted enzyme was interconvertible between the type D and 0 forms with SH reagents in the same way as native enzyme. In parallel experiments, dithiothreitol-treated deflavoenzyme was also reconstituted with FAD as described above. The activity of reconstituted enzyme, measured aerobically at 295 nm in the presence of NAD⁺, was 80% of that of the original. The NAD-dependent activity of this enzyme suggested that 85% of the enzyme was type D (Table I, Condition 3). These data show that deflavoenzyme can incorporate FAD to recover activity, 80–85% of that of the original, regardless of whether or not it undergoes DTT treatment before reconstitution, but that DTT treatment of deflavoenzyme is possibly more effective than that of reconstituted enzyme in the reduction of disulphides to sulphhydryl groups, which are responsible for interconversion between type O and D enzymes (Table I, Conditions 2 and 3).

Enzyme Reconstitution with 8-Mercapto-FAD—Fig. 2 shows the spectrum of enzyme reconstituted from deflavoenzyme and 8-mercapto-FAD without DTT treatment. The reconstituted enzyme had an absorption peak at 568 nm and an extinction coefficient of 41.200 M⁻¹ cm⁻², which is very similar to that of 8-mercapto-FAD milk xanthine oxidase.

### Table I

Catalytic activities of flavin-reconstituted rat liver deflavoxanthine dehydrogenase

| Flavin         | Conditions                                      | Activity               |
|----------------|-------------------------------------------------|------------------------|
|                |                                                  | NAD-dependent | O₂-dependent | NAD- and O₂-dependent |
|                |                                                  | mol/min/mol subunit | mol/min/mol subunit | mol/min/mol subunit |
| FAD            | 1) After reconstitution of DTT-untreated defla- | 0           | 614          | 630                   |
|                | voenzyme                                        | 2) DTT treatment of  | 370          | 180                   |
|                |                                                  | Sample 1       | 510          | 86                    |
|                | 3) After reconstitution of DTT-treated defla-   | 0           | 510          | 510                   |
|                | voenzyme                                        | 4) After reconstitution of | 370          | 120                   |
|                |                                                  | DTT-untreated defla- | 5) After reconstitution of | 90       | 370                   |
|                |                                                  | voenzyme       | DTT-treated defla- | 2 days on ice | 460 |
|                |                                                  | 6) Storage of Sample 5 for | 7) After reconstitution of | 0       | 180                   |
|                |                                                  | 2 days on ice  | DTT-untreated defla- | 210     | 210                   |
|                |                                                  | 8) After reconstitution of | 9) Storage of Sample 8 for | 210     | 230                   |
|                |                                                  | DTT-treated defla- | 20 days on ice | 230     | 230                   |
| 8-Mercapto-FAD |                                                  | 9) Storage of Sample 8 for | 20 days on ice | 230     | 230                   |
| 6-OH-FAD       |                                                  | 10) Storage of Sample 10 for | 10 days on ice | 100     | 100                   |

Activities are shown of enzyme reconstituted by incubating DTT-untreated and -treated deflavoenzymes with FAD and its artificial analogues. DTT-untreated and -treated deflavoenzymes were incubated with FAD, 8-mercapto-FAD, and 6-OH-FAD as described under "Materials and Methods." NAD-dependent activity was determined aerobically following absorbance at 340 nm in the presence of NAD⁺ (except for that of Sample 6, which was estimated from the absorbance changes at 295 nm in the presence and absence of NAD). O₂-dependent activity was determined aerobically following absorbance at 295 nm in the absence of NAD. NAD- and O₂-dependent activity was measured aerobically at 295 nm in the presence of NAD. All activities are expressed as relative turnover number under standard assay conditions, which is corrected for the activity-to-flavin ratio of original native enzyme (135, 130 and 131 in the cases of normal FAD, 8-mercapto-FAD, and 6-OH FAD enzymes, respectively) assuming that the activity-to-flavin ratio of fully active enzyme is 200.
residues essential for the type D-to-0 conversion still oxidized even after DTT treatment (about 28%, calculated from absorbance at 568 nm). This is consistent with the result of $E_{428} = 15,900 \text{ M}^{-1} \text{ cm}^{-1}$ of enzyme-bound 8-mercapto-FAD, which is 76% of that of free neutral 8-mercapto-flavin (21,000 M$^{-1} \text{ cm}^{-1}$ at this wavelength) (Moore et al., 1979). The NAD-dependent activity of reconstituted enzyme is 3.1 times higher than the O$_2$-dependent activity, which is also consistent with the spectral change. The sum of the two activities is almost the same as that of 8-mercapto-FAD enzyme reconstituted from deflavoenzyme without DTT treatment (Table I, Condition 5).

Fig. 4 shows the spectrum of 8-mercapto-FAD enzyme freshly reconstituted from DTT-treated deflavoenzyme and that of the same sample recorded after storage on ice for 2 days. In the latter spectrum, the peak at 450 nm is depressed, and the peak at 568 nm is raised. These results suggest that a portion of enzyme-bound 8-mercapto-FAD which had existed as the neutral form immediately after reconstitution had been converted to the anionic form, presumably as a result of autoxidation of sulfhydryl groups in the enzyme protein. Such spontaneous conversion of type D to O enzyme also occurs with native enzyme (Waud and Rajagopalan, 1976a). Accompanying the changes shown in Fig. 4, O$_2$-dependent activity was increased to 3.1 times that measured immediately after reconstitution (Table I, Condition 6).

In a comparison experiment, 8-mercapto-FAD enzyme reconstituted without prior DTT treatment was incubated with 5 mM DTT at 20 °C for 7 h. After incubation, the enzyme showed appreciable NAD-dependent activity (46% of that of enzyme reconstituted with DTT-treated deflavoenzyme and 8-mercapto-FAD). After this incubation, the ratio of the absorption peak at 450 nm to that at 568 nm was increased from 0.77 to 1.26, consistent with this partial conversion (data not shown).

**Enzyme Reconstitution with 6-OH-FAD**—As shown in Fig. 5, the spectrum of enzyme reconstituted with 6-OH-FAD and deflavoenzyme in the absence of DTT has peaks at 320 and 428 nm, with $E_{428} = 42,500 \text{ M}^{-1} \text{ cm}^{-1}$. Enzyme-bound 6-OH-FAD is clearly in the anionic form because its difference spectrum has peaks at 320 and 600 nm in addition to that at 428 nm, as in the case of 6-OH-FAD milk oxidase (Mayhew et al., 1974; Hille et al., 1981; Massey et al., 1989). The extinction coefficient at 428 nm of enzyme-bound 6-OH-FAD is calculated to be 12,200 M$^{-1} \text{ cm}^{-1}$, which is comparable to $E_{478} = 22,600 \text{ M}^{-1} \text{ cm}^{-1}$ of free anionic 6-OH-FAD (Mayhew et al., 1974). Reconstituted enzyme showed only O$_2$-dependent activity after removal of excess flavin (-----) and after storage for 2 days on ice (---).
Analyses of deflavoenzyme activities and flavin interactions with the enzyme. (A) Spectral analysis of deflavoenzyme activity and redox states. Enzyme activity, which is 29% of that of normal FAD enzyme reconstituted from deflavoenzyme not treated with DTT (Table I, Condition 7). The spectrum of enzyme reconstituted with 6-OH-FAD and DTT-treated deflavoenzyme has an absorption maximum at 420 nm and $E_{420} = 43,900$ M$^{-1}$ cm$^{-1}$ (Fig. 6). The difference spectrum of enzyme-bound 6-OH-FAD also shows an absorbance peak at 420 nm ($E_{420} = 20,800$ M$^{-1}$ cm$^{-1}$) and lacks a discrete maximum in the 600 nm region, suggesting its binding in the neutral form, as in the case of 6-OH-FAD chicken liver dehydrogenase (Nishino et al., 1989b). The NAD-dependent activity of reconstituted enzyme is 3.6 times higher than the O$_2$-dependent activity. The sum of the two activities is 1.2 times that of 6-OH-FAD enzyme reconstituted from deflavoenzyme without DTT treatment (Table I, Condition 8).

(B) Time-dependent spectral changes of enzyme reconstituted with 6-OH-FAD from DTT-treated deflavoenzyme. The absorbance spectrum of 6-OH-FAD enzyme reconstituted from DTT-treated deflavoenzyme was recorded immediately after removal of excess flavin (———) and after storage for 20 days on ice (——). Fig. 7 shows the spectrum of 6-OH-FAD enzyme reconstituted from DTT-treated deflavoenzyme directly after preparation and that of the same sample recorded after storage on ice for 20 days. In the latter spectrum, the peak at 320 nm is raised, and the absorbance around 600 nm is also raised. Moreover, the peak at 421 nm of the former spectrum is shifted to 428 nm. These changes show that some of the enzyme-bound 6-OH-FAD which had existed as the neutral form immediately after reconstitution became anionic during storage, presumably due to autoxidation of sulfhydryl groups in the enzyme protein. After storage, the enzyme showed only O$_2$-dependent activity, coincident with the spectral change (Table I, Condition 9).

6-OH-FAD enzyme reconstituted from DTT-untreated deflavoenzyme was incubated with 5.7 mM DTT at 15°C overnight. After incubation, the enzyme showed only a low xanthine:NAD reductase activity (41% of O$_2$-dependent activity). This partial conversion was not correlated with any clear change in the spectrum of the enzyme (data not shown).

**Discussion**

In this work, rat liver deflavoxanthine dehydrogenase was prepared that can be reconstituted with FAD to recover activity close to that of the original enzyme. The activity of holoenzyme reconstituted from DTT-untreated deflavoenzyme and FAD is totally O$_2$-dependent, like the native type O enzyme, and that from DTT-treated deflavoenzyme and FAD is mostly NAD-dependent, like the native type D enzyme. Thus, the single apoenzyme can provide two different flavin environments which are interconvertible through treatment with SH reagents. It has been shown that when the native type D enzyme was converted to the type O enzyme by reaction with 4,4'-dithiodipyrindine, about 8 mol of 4-thiopyridone were formed per mol of FAD after addition of 4 mol of 4,4'-dithiodipyrindine/mole of FAD (Saito, 1987). This indicated that cysteinyl residues were located close enough to each other to form disulfide bonds and that, at most, 8 residues were oxidized during conversion from the type D to O enzymes as discussed previously (Saito, 1987).

These two interconvertible rat liver deflavoenzymes were also successfully reconstituted with 8-mercapto-FAD and 6-OH-FAD. In these cases, the catalytic behavior of the artificial flavin enzymes is clearly different between DTT-treated and -untreated enzymes, i.e. DTT-untreated and -treated defla-
voenzymes showed totally $O_2$-dependent and mostly NAD-dependent xanthine oxidizing activities upon reconstitution, respectively. The effect of DTT treatment is more pronounced in the apoenzyme than in the reconstituted enzymes, suggesting that cysteinyl residues responsible for the type D-to-O conversion are more open to solvent-borne reagents in the apoenzyme than in the reconstituted enzymes. The 8-mercapto-FAD and 6-OH-FAD enzymes reconstituted from untreated deflavoenzymes showed only little NAD-dependent activity after incubation with DTT as compared with enzyme reconstituted from DTT-untreated deflavoenzyme and normal FAD. The possible reason for this might be lower accessibility in these artificial enzymes than in native enzymes of DTT to the cysteinyl residues responsible for the conversion.

Differences in the flavin environment of the reconstituted enzymes were also clear from the ionization state of the enzyme-bound flavins. The two FAD analogues bound to DTT-untreated deflavoenzyme are in the anionic form with a predominant resonance form having the negative charge in the flavin N(1)-C(2)=O region, suggesting that some amino acid residues of the protein should serve as a stabilizer of this structure, for example, a positively charged residue near the N-1 position of the isoxalazine ring (Massey et al., 1979). In contrast, in DTT-treatment enzyme, the two FAD analogues are in the neutral form, with some protein interactions making the $pK_a$ values of the sulfhydryl group of 8-mercapto-FAD ($pK_a = 3.8$ in free system; Moore et al., 1979) and the hydroxyl group of 6-OH-FAD ($pK_a = 7.1$ in free system, Mayhew et al., 1974) higher than 8.0.

It was previously shown through active site probe studies that the environment of flavin is different in chicken liver xanthine dehydrogenase and milk xanthine oxidase. As these enzymes are quite similar to rat liver type D and O enzymes, respectively, in kinetic and redox properties, it was obviously of considerable interest to determine whether a similar difference in flavin environment would be observed in the two types of rat liver enzymes. In this work, it was demonstrated that the two different protein environments around the flavin found in chicken liver xanthine dehydrogenase and milk xanthine oxidase are indeed present interconvertibly on a single enzyme. DTT-treated rat liver enzyme, whose activity is mostly NAD-dependent, has a flavin-binding pocket similar to that of the chicken liver dehydrogenase; and untreated rat liver enzyme, whose activity is totally $O_2$-dependent, has one similar to that of the milk oxidase. The possible changes in the protein environment have been discussed in more detail a previous paper (Massey et al., 1989).

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