Chemical Modification of the N-10 Ribityl Side Chain of Flavins

EFFECTS ON PROPERTIES OF FLAVOPROTEIN DISULFIDE OXIDOREDUCTASES*

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Three flavin derivatives modified at the 2'-position of the flavin N-10 ribityl side chain were synthesized: arabinoflavin, 2'-F-2-deoxyarabinoflavin, and 2'-deoxyriboflavin. These were converted to the FAD level with FAD synthetase. Apoproteins of lipoamide dehydrogenase, glutathione reductase, and mercuric reductase, a family of flavoprotein oxidoreductases, were reconstituted with these flavins. Significant reduction of the catalytic activities was observed with the modified enzymes. During anaerobic reduction of the modified enzymes with substrate or dithiothreitol, decreased thermodynamic stability of the two-electron reduced enzyme forms (E1H2) and the accumulation of the four-electron reduced forms (E1H4) noted. This effect was more pronounced in case of arabinoflavin-reconstituted enzymes than with the other two. It was found that NAD+ binding influences the interaction between the flavin and the reduced disulfide in the 2'-F-arabinoflavin-FAD-lipoamide dehydrogenase, presumably by altering the relative oxidation-reduction potentials. 19F NMR data were obtained for different forms of the 2'-F-deoxyarabinoflavin, 2'-deoxyriboflavin, and 2'-deoxyarabinoflavin. These were converted to the FAD level with FAD synthetase. Significant reduction of the relative oxidation-reduction potentials.19F NMR data were obtained for different forms of the 2'-F-arabinoflavin-FAD-reconstituted mercuric reductase (9, 39) showed that the two-electron reduced form in which the flavin remains oxidized and the active site disulfide reduced is destabilized by this modification and complete flavin reduction was observed. The crystal structures of glutathione reductase and lipoamide dehydrogenase (3, 6) have shown that the 2'-hydroxyl group is involved in an important hydrogen bond network in the oxidized forms of the proteins. The 2'- and 4'-hydroxyls and an adenine phosphate oxygen atom make two intramolecular hydrogen bonds that could play a significant role in determining the conformation of the bound prosthetic group (Fig. 1A). In the reduced form, the distance between 2'-OH and the active site Cys-63 decreases from 3.9 to 3.4 Å. Also Sol-601 now donates hydrogen bonds to the phosphate oxygen and 4'-OH, so that the 2'-OH can rotate its hydrogen around to stabilize the active site thiolate anion through a hydrogen bond, making it possible to accommodate a buried charge generated during catalysis (Fig. 1B). These literature reports clearly highlight the possible importance for the 2'-hydroxyl group in regulating protein structure and catalysis.

Lipoamide dehydrogenase, glutathione reductase, and mercuric reductase represent a well studied family of flavoprotein-disulfide oxidoreductases. The catalytic reaction mechanisms of each of them present a number of common features (10). The flow of electrons during catalysis in these enzymes is to or from pyridine nucleotide to FAD to active center disulfide to substrate disulfide. All three of these proteins exist in three different redox forms, i.e. oxidized (E0), two electron reduced (E1H2), and four electron reduced (E1H4) states, the E1H2 form being catalytically relevant. In the light of known crystal structures, these proteins constitute an excellent set of models for 19F NMR as well as enzymatic studies with 2'-position-modified flavins. We were interested to see how the different oxidation states of these proteins differ structurally from one another by 19F NMR chemical shifts and also how the chemical modifications may affect catalysis. Accordingly, we synthesized three flavin derivatives modified at the 2'-position of the ribityl side chain i.e. 2'-deoxyriboflavin, arabinoflavin, and 2'-F-arabinoflavin.

The fluoroflavin has the potential of being a useful 19F NMR probe. Such NMR studies employing 8-fluoroflavins provided a wealth of information on the structure, conformation, and dynamics of flavoproteins (11). Of all the nuclei, 19F is one of the most effective ones because of its high abundance, extreme sensitivity to the surrounding environment, and a large range of chemical shifts (12-14). Furthermore, since the proteins normally do not contain any fluorine, one can investigate protein-prosthetic group, prosthetic group-ligand interactions without interference from the protein. The potential relevance of these modified flavins is also highlighted by some recent work with p-hydroxybenzoate hydroxylase. The flavin in this

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NMR Sample Preparation

Protein samples for NMR were concentrated by means of a Centricon 30 microconcentrator to 150–200 μl and were diluted to 400 μl with 50 μl of D_{2}O and for the rest of the volume with buffer.

Reduced flavin and protein samples were prepared by the addition of known excess of reducing agent to sample in the NMR tube. The tubes were flushed with argon (space over the sample) for 15 min prior to the addition of the reductant in buffer. Argon flushing was continued during the addition. The tube was then closed with a standard plastic cap and sealed with parafilm. Color changes associated with the reduction persisted for several days.

NMR Acquisition Parameters

Fluorine NMR spectra were recorded with a 10-mm 19F probe and with a General Electric GN 500 instrument, operating at 470 MHz. All of the NMR measurements were carried out without proton decoupling, at 25 °C (23). Fluorine chemical shifts were measured by using hexafluorobenzene as an external standard and are given in parts/million. Spectra were measured with 16,000 or 32,000 data points over spectral widths from 20 to 45 kHz. The repetition time was 0.005 to 1 s with a 12-μs pulse (60° flip angle).

NMR Processing

With broad lines and low signal to noise taken over a wide spectral width, the base line of an NMR spectrum is not flat (24, 25). Correction routines (GEM routines IC, FB, and BF) were used to flatten the base line. Exponential line broadening was performed from 10 to 50 Hz.

Syntheses of Modified Flavins

The fluoroorabinaflavin was synthesized (Scheme 1) by making use of the Tishler condensation reaction (26). 2-Fluoro-2-deoxyarabinose—1 g of 2-fluoro-1,3,4,5-tetra-O-benzoyl-arabinose was dissolved in 40 ml of methanol + ammonium hydroxide mixture (1:1) and stirred overnight with a magnetic stirrer. The solvents were then evaporated with a rotary evaporator, and the obtained solid was dissolved in a minimum volume of methanol and loaded on a (1.5 × 8 cm) silica gel column. The benzoic acid and benzamide products were eluted with a mixture of ethyl acetate and hexane (3:1). Washing the column with 100% methanol and evaporation of the solvent gave the debenzoylated fluoro sugar in 82% (272 mg) yield.

N-2-F-2-deoxyarabinol-3,4-dimethyl Benzimine—ortho-Xylylene (194 mg) and 245 mg of the fluoroarabinase were dissolved in 15 ml of ethanol and refluxed for 3 h. After cooling in a refrigerator, the crystals were filtered off and washed with cold ether to obtain 315 mg of fluoroorimine in 72% yield.

Reduction of the Imine—The fluoroorimine, 315 mg, obtained in the above reaction was dissolved in 25 ml of ethanol, and excess sodium borohydride (400 mg) was added. After stirring the reaction mixture overnight, the excess reagent was destroyed with the careful addition of water, the solvent was evaporated, and the solid was diazotized without purification.

Diazotization—185 mg of freshly distilled aniline was dissolved in 2 ml of acetic acid, and the solution was diluted with 2 ml of water. Then, 1 ml of concentrated HCl was added dropwise with cooling. To this solution, 170 mg of solid NaN_{3} was added while maintaining the temperature at less than 5 °C. The so-formed diazonium chloride was kept at 0 °C for at least 5 min for its complete formation. At this stage, the solid obtained in the preceding reaction was suspended in a minimum volume of acetic acid and cooled to 0 °C. The precolored azo dye was added dropwise to the acetic acid solution of amine, and the temperature was kept at 0 °C for 30 min. A concentrated solution of sodium hydroxide (approximately 400 mg in 1 ml water) was added and care was taken that the pH remained below 4 (~3–4) after the addition. The reaction mixture was kept at 0 °C for 2 h. The reaction mixture was then extracted with 100 ml of ether, and the ether layer was separated. The aqueous solution was reextracted twice with 50 ml each of ether, and the combined organic layers were washed with saturated sodium bicarbonate to neutralize acid and washed with water following by drying over anhydrous sodium sulfate. Evaporation of the ether gave 186 mg of azo dye (45% yield from the imine).

2'-F-2'-deoxyarabinoflavin—185 mg of the azo dye and 70 mg of barbituric acid were suspended in 10 ml of n-butyl alcohol. To this mixture, 2.5 ml of acetic acid was added and refluxed for 3 h. Thin-layer chromatography on silica gel (developed in ethyl acetate) at this stage showed no azo dye, and a yellow fluorescent spot was observed at the base. Development of the thin-layer chromatography in 30% methanol
anRP-18 column eluted with a mixture of 80% 0.01M potassium Pi, pH starting riboflavin was detected. The FAD was purified by HPLC over converted to the FAD with around 25% contamination by the FMN. No used for a further 24 h. After the second day, 70% of the riboflavin was the addition of a second lot of FAD synthetase, incubation was contin-
mixtures showed around 40% conversion to FAD and 50% to FMN. After
in chloroform showed a yellow fluorescent spot at Rf value of 0.7. Normal butanol was removed by rotary evaporation, and the solid was purified by preparative HPLC1 (isocratic system of 60% 0.01M amino-
noium formate and 40% methanol over a Partisil C8 column).
Analytical Data for the Fluoroarabinoflavin—1H NMR data (200 MHz, Me4SO-d4/trimethylsilane): 6.83 (s, 1H, amide proton), 7.85 (s, 1H, aromatic proton), 7.65 (s, 1H, aromatic proton), 5.5–6.5 (ribo-
tyl side chain protons), 2.4 (s, 3H, aromatic methyl), 2.3 (s, 3H, aromatic methyl).
Positive Ion FAB Mass Spectrometry Data—(M+ + 1): 379. 19FNMR (500 MHz, Me4SO-d4/CD3F): 66.8 ppm.
Conversion of 2'-F-arabarainoflavin to the FAD and FMN Level—Flu-
oroarabinoflavon was converted to the FAD level with partially purified FAD synthetase from Brevibacterium ammoniagenes by incubating the flavin in 0.05M potassium Pi, pH 7.5, at 37°C, following the procedure of Spencer et al. (27). After 24 h, HPLC analysis of the incubation mixture showed around 40% conversion to FAD and 50% to FMN. After the addition of a second lot of FAD synthetase, incubation was continued for a further 24 h. After the second day, 70% of the riboflavin was converted to the FAD with around 25% contamination by the FMN. No starting riboflavin was detected. The FAD was purified by HPLC over an RP-18 column eluted with a mixture of 80% 0.01M potassium Pi, pH 6, and 20% methanol. 2'-F-arabarainofMN was obtained by hydrolysis of the FAD in 0.1M potassium Pi, pH 7, with snake venom phosphodies-
terase (Naja naja venom).
Synthesis of 2'-Deoxyarabinoflavin—The 2'-deoxy riboflavin was synthe-
sized by the same sequence of reactions described for the 2-fluoro-
arabinoflavin (Scheme 1). In this case 2'-deoxyriboseline was used in reaction with 3,4-dimethyl aniline as the first step. Hydrogenation of the Schiff base in the next step was carried out at room temperature and pressure over Pd/C. The yields of all the reactions fell in the same ranges as for the 2'-fluoroflavin. HPLC-pure 2'-deoxyarabinoflavin was taken to the FAD level with the partially purified FAD synthetase, again under the same conditions used for the fluoroflavin. After the second day of incubation, the riboflavin was converted almost comple-
tely to the FAD level. 2'-deoxy-FMN was obtained by treating the HPLC pure 2'-deoxy-FAD with snake venom.
Positive Ion FAB Mass Spec Data for 2'-Deoxyriboflavin—(M+ + 1): 361.
Synthesis of Arabinoflavins—Arabinoflavin was synthesized, again by making use of the above method (Scheme 1). In this case 3,4-arabinose was the sugar used in the first condensation reaction with 3,4-dimethyl aniline. However the reduction of the Schiff base obtained in this

\[\text{Scheme 1. Synthesis of 2'-fluoro-2'-deoxyarabinoflavin.}\]

Reagents: (1) NH2OH, MeOH, RT, 24 hrs. (2) 3,4- Dimethylaniline, MeOH, 3 hrs, Reflux. (3) 4 eq. of NaBH4, MeOH, 24 hrs. (4) C6H5N2Cl. AcOH, 0°C, 2hrs. (5) Barbituric acid, AcOH, n-Butanol, 3 hrs, Reflux.

RESULTS AND DISCUSSION

Fluorescence Properties of the Free Flavins

An interesting descending trend was observed when quantitative measurements of the fluorescence of the four FAD mol-
ecules, namely normal FAD, 2'-F-arabarainof-FAD, arabarainof-FAD, and 2'-deoxy-FAD, were carried out at the same concentration. The order of fluorescence intensity was determined to be normal FAD > 2'-F-arabarainof-FAD — arabarainof-FAD > 2'-deoxy-FAD (1.7:1.4:1.4:1). It is known that the intramolecular stacking of the adenine moiety on the isoalloxazine ring of the flavin decreases the fluorescence of FAD when compared with riboflavin or FMN (28, 29). When we made space filling models for these FAD molecules, it was noted that the substituent at the 2'-
carbon restricts the extent of stacking of the adenine moiety on the isoalloxazine ring. The results suggest a stronger intramo-
lecular complex between the adenine and isoalloxazine for the 2'-deoxy-FAD, intermediate for 2'-F-arabarainof-FAD, or arabarainof-FAD and comparatively weaker for normal FAD, in keeping with the size of the substituent at the 2'-position. This was further substantiated by converting all of the FAD level mol-
ecules to the FMN level with snake venom and measuring the fluorescence. It was found that the increase in fluorescence from FAD to FMN for these flavins was 21.5 times for 2'-
deoxyflavin, 16.9 times for 2'-F-flavin, 17 times for arabarainof-flavin, and 11.3 times for normal flavin. This is a very clear experimental demonstration of the effect on fluorescence of intramolecular complex formation between the adenine and isoalloxazine rings in FAD molecules.

1 The abbreviations used are: HPLC, high performance liquid chromato-
graphy; DTT, dithiothreitol; FAB, fast atom bombardment.
Redox properties of the flavin and the catalytic properties of flavoproteins are known to be related. Since we reconstructed the above flavins into a number of flavoproteins, the measurement of potentials is of obvious interest. The potentials were measured for 2'-deoxy-FAD and arabinoflavin by using the xanthine/xanthine oxidase system and anthraquinone-2-sulfonate as the reference dye (30). Reduction of the deoxy-FAD has an isosbestic point at 336 nm, and reduction of the dye has an isosbestic point at 354 nm. These wavelengths were used to monitor the reduction of the components in a mixture of the two. A plot of log([ox]/[red]) of the dye against log([ox]/[red]) of the flavin gave a midpoint potential for 2'-deoxy-FAD at -219.5 mV, slightly more negative than that of normal FAD (220 mV; Ref. 30). Reduction of the arabinoflavin has also shown an isosbestic point at 336 nm. A similar plot for the arabinoflavin showed a potential for arabinoflavin of -207 mV, identical to the value of native FAD. The 2'-fluoroflavins was also reported to have the same potential as the normal flavin (9).

$^{19}$F NMR Spectra of Free Flavins

A single peak was recorded for oxidized 2'-F-riboflavin at 66.3 ppm in 0.1 M potassium P$_2$O$_5$, pH 7. When the spectrum was recorded for the oxidized form of the free flavin in dimethyl sulfoxide, a single resonance at 66.8 ppm was recorded. This suggests very little or no solvent effect on the fluorine chemical shift, going from protic to aprotic solvent. Sodium dithionite-reduced 2'-F-riboflavin has a signal at 65.5 ppm, around 1 ppm upfield shift from the oxidized form. It shows, as expected, that reduction of the isoalloxazine ring has very little effect on the fluorine resonance. This is unlike the case of 8-F-flavins, where the benzene ring of the flavin experiences more positive character due to the presence of the electronegative fluorine (11) and results in large differences in the chemical shifts of the oxidized (65.4 ppm) and reduced (36.0 ppm) forms. The $^{19}$F NMR resonance of the oxidized 2'-F-arabino-FAD was seen at 65.2 ppm, one ppm upfield shift from that of 2'-F-arabinoflavin. This can be attributed to the intramolecular stacking of the adenine moiety on the isoalloxazine (28, 29). The sodium dithionite-reduced FAD has a peak at 66.1 ppm. The $^{19}$F resonances for the oxidized and reduced FMN were recorded at 66.5 and 67.2 ppm respectively (Table I).

Riboflavin Binding Protein

The 2'-fluororiboflavin binds to the apoprotein of hen egg white riboflavin binding protein, with a $K_a$ of 4.2 $\pm$ 0.3 $\times$ 10$^{-8}$ M at pH 7, 25 °C. By standardization of the apoprotein with pure riboflavin, the extinction coefficient at 444 nm of the 2'-fluororiboflavin was determined as 11,400 $\times$ 10$^{-3}$ cm$^{-1}$. The extinction coefficients for 2'-F-arabino-FMN and 2'-F-arabino-FAD were determined as 11,300 and 10,800 $\times$ 10$^{-3}$ cm$^{-1}$, respectively. The free flavin has $\lambda_{max}$ at 372 and 444 nm. Upon binding to aporiboflavin binding protein the $\lambda_{max}$ shifted to 378 and 454 nm. The $^{19}$F NMR spectrum of the oxidized 2'-F-arabino-flavin bound to riboflavin binding protein showed a resonance at 66.0 ppm. This is the same chemical shift observed for the unbound free flavin, suggesting minimal protein-fluorine interactions. However, the increased line width of the signal from that of the free flavin confirms that the flavin is bound to the protein. When the reconstituted riboflavin binding protein was reduced with dithionite, a signal at 65.9 ppm was recorded, again in the same region as the free flavin. The narrow line width of the signal suggests the flavin may not be bound to the protein in its reduced state.

Reductive properties of the flavin and the catalytic properties of flavoproteins are known to be related. Since we reconstructed the above flavins into a number of flavoproteins, the measurement of potentials is of obvious interest. The potentials were measured for 2'-deoxy-FAD and arabinoflavin by using the xanthine/xanthine oxidase system and anthraquinone-2-sulfonate as the reference dye (30). Reduction of the deoxy-FAD has an isosbestic point at 336 nm, and reduction of the dye has an isosbestic point at 354 nm. These wavelengths were used to monitor the reduction of the components in a mixture of the two. A plot of log([ox]/[red]) of the dye against log([ox]/[red]) of the flavin gave a midpoint potential for 2'-deoxy-FAD of -219.5 mV, slightly more negative than that of normal FAD (220 mV; Ref. 30). Reduction of the arabinoflavin has also shown an isosbestic point at 336 nm. A similar plot for the arabinoflavin gave a potential for arabinoflavin of -207 mV, identical to the value of native FAD. The 2'-fluoroflavins was also reported to have the same potential as the normal flavin (9).

$^{19}$F NMR data including free and protein-bound fluoroflavins

Spectra were recorded in 100 mM potassium P$_2$O$_5$, pH 7, with 10% D$_2$O and hexafluorobenzene as an external standard. Buffer contained 0.5 mM EDTA in case of protein samples, except for riboflavin binding protein.

### TABLE I

| Compound                      | Oxidized | Reduced |
|-------------------------------|----------|---------|
| 2'-F-arabinoflavin (buffer)   | 66.3     | 65.5    |
| 2'-F-arabinoflavin (Me$_2$SO) | 66.8     |         |
| 2'-F-arabino-FMN              | 66.5     | 67.2    |
| 2'-F-arabino-FAD              | 65.2     | 66.1    |
| Riboflavin binding protein    | 66.0     | 65.9    |
| Lipoamide dehydrogenase       | 72.3     |         |
| Lipoamide dehydrogenase + 3 eq of NADH | 79.1    |         |
| Lipoamide dehydrogenase + 12 eq of NADH | 64.8 |         |
| Lipoamide dehydrogenase + 4 eq of dihydrolipoamide | 72.0 |         |
| Lipoamide dehydrogenase + 3 eq of NADPH | 71.3 |         |
| Lipoamide dehydrogenase + excess dithionite | 67.8 |         |
| Glutathione reductase         | 73.0     |         |
| Glutathione reductase + DTT   | No signal|         |
| Glutathione reductase + DTT + dithionite | 46.94 |         |
| Potassium fluoride            | 47.3     |         |
| Mercuroic reductase           | 72.3     |         |
| 4-F-phenol                    | 41.6     |         |
| 2,4-F$_2$-phenol              | 44.4, 33.3|        |

Reconstitution of Apolipoamide Dehydrogenase with Modified Flavins

A small excess (1.5 equivalents) of pure modified flavin was added to the 50% ethylene glycol solution of apoprotein and incubated for 24 h at 4 °C. Then, the excess flavin and ethylene glycol were removed by ultrafiltration with a Centricon 30 microconcentrator and equilibration with 100 mM potassium P$_2$O$_5$, pH 7, containing 0.3 mM EDTA. The reconstituted proteins have well resolved absorption spectra indicative of an apolar environment, as in the case of the native enzyme. Comparatively larger shifts from the free flavin to bound flavin were observed in the spectral region of the near UV peak (Table II) in all cases.

Reduction of Reconstituted Lipoamide Dehydrogenase Forms with Dihydrolipoamide and the Stability of the $E_{H2}$ Form—Native pig heart lipoamide dehydrogenase is reduced rapidly and almost stoichiometrically by the substrate to the $E_{H2}$ form, and even the use of excess dihydrolipoamide leads to no further reduction of the $E_{H2}$ form. Dithionite is required for complete reduction of $E_{H2}$ to $E_{H4}$. Since $E_{H2}$ is the catalytically relevant species, the effects of the present modifications on the stability of this form were studied by the anaerobic reduction of the reconstituted proteins with the substrate (Fig. 2). The typical $E_{H2}$ form is represented by a charge transfer complex with typical long wavelength absorption in which the thiolate of Cys-63 is the donor and FAD is the acceptor (31).

Arabino-FAD-Lipoamide Dehydrogenase—The spectrum recorded immediately after the anaerobic addition of 1.6 eq of dihydrolipoamide to the reconstituted protein showed little or no charge transfer band typical of the $E_{H2}$ form, with about 50% reduction in the flavin absorption bands. After this spectrum was stable, addition of another 4 eq of substrate resulted in complete reduction; the spectrum recorded is typical of the $E_{H4}$ form (Fig. 2A).

2'-F-Arabino-FAD-Lipoamide Dehydrogenase—The absorption spectrum of the reconstituted protein with 1.6 eq of substrate, showed somewhat less long wavelength absorbance
than is typical for the EH\textsubscript{2} form. With the addition of another 6 eq of substrate, further reduction of the flavin absorption was observed, but with the development of more long wavelength absorbance (Fig. 2B).

2'-Dexoxy-FAD-Lipoamide Dehydrogenase—With 16 eq of substrate, the enzyme was reduced to the EH\textsubscript{2} form with the typical charge transfer band. Further addition of another 6 eq of dihydrolipoamide resulted in the slow reduction of the flavin absorption peaks with a decrease in the charge transfer band, suggesting a small reduction to the EH\textsubscript{2} form (Fig. 2C).

These results suggest that the modifications at the 2'-position have affected the stability of the EH\textsubscript{2} form of the protein, presumably by changing the relative redox potentials of the bound flavin and the active site disulfide/dithiol couple. The effect is clearly most dramatic with arabino-FAD enzyme.

Reduction of 2'-F-arabinolipoamide Dehydrogenase with NADH—For the native enzyme, the spectrum when reduced by stochiometric NADH is slightly different from that when reduced by dihydrolipoamide. This is because of the formation of an EH\textsubscript{2}-NAD\textsuperscript{+} complex when NADH is the reductant (32). 2'-F-arabino-FAD-reconstituted protein was reduced anaerobically with NADH (Fig. 3A). The spectrum resulting from the addition of one equivalent of NADH shows much less absorbance in the 530 nm region than the typical EH\textsubscript{2} form of the enzyme. The extent of bleaching at 450 nm is indicative of the presence of an appreciable amount of flavin in the fully reduced form. After no further changes were observed spectrally, another 2 eq of NADH was added. This resulted in the complete reduction of the flavin, and the charge transfer band of reduced flavin with NADH\textsuperscript{+} (33) was observed.

Reduction of 2'-F-arabino-FAD Dehydrogenase with NADPH—Lipoamide dehydrogenase is very selective for

**Table II**

| Flavin Location             | Free Flavin | Lipoyamide Dehydrogenase | Glutathione Reductase | Mercuric Reductase |
|-----------------------------|-------------|--------------------------|-----------------------|-------------------|
| Arabino-FAD                 | 376,448     | 346,452                  | 378,458               | 366,450           |
| 2'-Dexoxy-FAD               | 374,446     | 344,452                  | 370,460               | 364,452           |
| 2'-F-arabino-FAD            | 372,444     | 348,456                  | 366,460               | 352,456           |

**Fig. 2. Reduction of lipoamide dehydrogenases with dihydrolipoamide.** Reactions were carried out anaerobically in 100 mM potassium P\textsubscript{i}, pH 7, containing 0.5 mM EDTA at 25 °C. A, reduction of 2'-F-arabino-FAD-lipoamide dehydrogenase with dihydrolipoamide. ---, oxidized enzyme; --, after addition of 1 eq of dihydrolipoamide; A, after 6 eq of dihydrolipoamide. B, reduction of arabino-FAD-lipoamide dehydrogenase with dihydrolipoamide. ---, oxidized enzyme; --, after 1.6 eq of dihydrolipoamide; --, after 6 eq of dihydrolipoamide. C, reduction of 2'-Dexoxy-FAD-lipoamide dehydrogenase with dihydrolipoamide. ---, oxidized enzyme; --, after 1.6 eq of dihydrolipoamide; --, after 6 eq of dihydrolipoamide.

**Fig. 3. Anaerobic reduction of 2'-F-arabinolipoamide dehydrogenase with NADH and NADPH.** Reactions were carried out in 100 mM potassium P\textsubscript{i}, pH 7, containing 0.5 mM EDTA at 25 °C. A, reduction with NADH. ---, oxidized enzyme; --, after addition of 1 eq of NADH; ---, after addition of another 2 eq of NADH. B, reduction with NADPH. ---, oxidized enzyme; --, adding 2 eq of NADPH; --, after further addition of 1 eq of NADPH.
form to almost the free flavin region. Reduction with excess NADH results in the formation of reduced flavin in complex with NAD$^+$ and is characterized by the blue-green color of the complex (33). The results suggest that, unlike in the E or the E$_{H2}$ forms, the fluorine might not be interacting with the protein in the E$_{H4}$ form.

NADPH-reduced Protein—When the protein was reduced with dihydrolipoamide to obtain the spectrum for the uncomplexed E$_{H2}$ form, the NMR spectrum showed one broad signal between 71.5 and 72.5 ppm. Because of the problems of establishing strict anaerobiosis in the NMR experiments, there was a possibility that we might be obtaining the NMR spectrum of the oxidized form of the protein. Hence $^{19}$F NMR was recorded with the NADPH-reduced protein since it is known that E$_{H2}$ forms, the fluorine might not be interacting with the protein in the E$_{H4}$ form.

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Dithionite-reduced Protein—The NMR spectrum that we obtained by reducing the flouroarabino-FAD enzyme with excess NADH is that of the complex between reduced flavin and NAD$^+$. To see if the complex formation of E$_{H4}$ with NAD perturbed the $^{19}$F chemical shift, NMR was recorded for the ligand-free E$_{H4}$ form by reducing the protein with excess dithionite. Dithionite-reduced enzyme showed a single resonance at 67.8 ppm, around 3 ppm upfield shift from that of the complex (Fig. 4).

Steady State Kinetics—All three reconstituted forms of the protein showed parallel line double-reciprocal plots when the NAD concentration was varied over several concentrations of dihydrolipoamide, as in the case of the native enzyme (37). The results (Table III) show that the catalytic activity of the protein is seriously hampered by the present modifications at the 2$^9$-position of the ribityl side chain. The 2'-deoxy-FAD form of the protein was found to be relatively more active than both the arabino forms. Rapid reaction kinetics studies are planned to determine which steps in catalysis are altered in the modified enzymes.

Reconstitution of Apo glutathione Reductase

The apoprotein was reconstituted with the three modified flavins by incubating with 1.5 eq of the flavin for 90 min at 0 °C and then the excess flavin was washed off with 100 mM potassium P$_i$, pH 7 containing 0.3 mM EDTA over a Centricon 30 microconcentrator. Absorption spectra of the reconstituted proteins showed the shifts typical of the native protein from the free flavin. In distinction to lipoamide dehydrogenase, little or no shift in the near UV flavin peak was observed on binding to the protein (Table II).

Reduction of the Reconstituted Glutathione Reductase Forms with DTT and the Stability of the E$_{H2}$ Form—The spectrum of two-electron reduced glutathione reductase is very similar to that of lipoamide dehydrogenase, and represented by the typical charge transfer band. DTT reduces the native protein to its E$_{H2}$ form. The reconstituted proteins were reduced anaerobically with DTT, and reduction was followed spectrophotometrically (Fig. 6). Arabino-FAD-reconstituted protein showed a typical E$_{H2}$ spectrum immediately after mixing with DTT but was
The effect of DTT on the 19F NMR spectrum of free flavin—which was found to be stable to nucleophilic displacement reactions with sulfide, normal thiols, and reduction with dithionite. In the light of the above effect of DTT on the NMR spectrum of reconstituted glutathione reductase, the effect on the free flavin was studied in detail. The 19F NMR spectrum was immediately quenched on addition of DTT to the 2',F-arabino-FAD-glutathione reductase with DTT. ——, oxidized enzyme; ——, 15 s after the addition of DTT; ———, after 45 s; ———, after 90 s; ———, after 180 s. B, reduction of 2',F-arabino-FAD-glutathione reductase with DTT; ———, oxidized enzyme; ———, stable spectrum after the addition of DTT (final concentration 100 μM); ———, after the addition of excess DTT (final concentration 1 mM). C, reduction of 2',Deoxy-FAD-glutathione reductase with DTT. ———, oxidized enzyme; ———, stable spectrum after the addition of DTT (final concentration, 100 μM).

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**Table III**

Activities of the modified lipoamide dehydrogenase forms

|                     | $k_{cat}$ (min$^{-1}$) | $K_m$ (lip(BSH)NH$_2$) (μM) | $K_m$ (NAD) (μM) |
|---------------------|------------------------|-----------------------------|-----------------|
| Native lipoamide dehydrogenase | 33,000                  | 300                         | 200             |
| Arabino-FAD-lipoamide dehydrogenase | 2,100                   | 220                         | 100             |
| 2',F-arabino-FAD-lipoamide dehydrogenase | 1,800                   | 140                         | 13              |
| 2',Deoxy-FAD-lipoamide dehydrogenase | 4,900                   | 160                         | 120             |

a From Ref. 37.

**Fig. 6. Anaerobic reduction of glutathione reductases with DTT.** Reactions were carried out in 100 mM potassium Pi, pH 7, containing 0.5 mM EDTA at 25°C. A, reduction of arabino-FAD-glutathione reductase with DTT (final concentration, 100 μM). ———, oxidized enzyme; ———, 15 s after the addition of DTT; ———, after 45 s; ———, after 90 s; ———, after 180 s. B, reduction of 2',F-arabino-FAD-glutathione reductase with DTT; ———, oxidized enzyme; ———, stable spectrum after the addition of DTT (final concentration 100 μM); ———, after the addition of excess DTT (final concentration 1 mM). C, reduction of 2',Deoxy-FAD-glutathione reductase with DTT. ———, oxidized enzyme; ———, stable spectrum after the addition of DTT (final concentration, 100 μM).

It should be noted that free 2',F-arabino-FAD was found to be stable to nucleophilic displacement reactions with sulfide, normal thiols, and reduction with dithionite. Since it was not possible to observe the 19F NMR spectra of other fluoro compounds. Hence 19F NMR spectra of 4-F-phenol and 2,4-difluorophenol were recorded in the absence and presence of DTT. The 4-F-phenol shows one resonance at 41.6 ppm. When DTT was added, no effect was found on the NMR signal and no fluoride elimination was observed over the same time scale. To get more insight into this observation, the effect of DTT on the NMR spectrum of an aprotic solvent was also studied. When DTT was added to the flavin in Me$_2$SO, neither was there any effect on the NMR spectrum nor was there any fluoride release. This clearly suggests that DTT in its ionic form might be complexing with the flavin side chain, thereby facilitating the release of fluoride by another DTT molecule. This was further supported by repeating the aqueous experiment at pH 10.5, where the effect was more pronounced and fluoride elimination was relatively faster. The signal for the released fluoride was observed within a few minutes, unlike at pH 7, where it took a few hours. With mercaptoethanol as the nucleophile at this pH, very slow release of fluoride was observed over a period of a few days.

In the light of the above observations, it was of obvious interest to check if DTT has a similar effect on the 19F NMR spectra of other fluoro compounds. Hence 19F NMR spectra of 4-F-phenol, 2,4-difluorophenol were recorded in the absence and presence of DTT. The 4-F-phenol shows one resonance at 41.6 ppm. When DTT was added, no effect was found on the NMR signal and no fluoride release was observed over a period of a few hours. The 2,4-difluorophenol has two peaks at 44.4 and 33.3 ppm, and again DTT addition resulted in no effect on the spectrum and no fluoride elimination.

Further Reduction of the DTT-reduced 2',F-arabino-FAD-glutathione Reductase with Dithionite Elimination of Fluoride—Since it was not possible to observe the 19F NMR spectrum for the DTT-reduced protein, it was further reduced to EH$_2$ with dithionite, and the NMR spectrum was recorded again. Interestingly, slow elimination of fluoride was observed with no signal visible for the flavin. When the protein was denatured with SDS, no flavin covalently bound to the protein was observed, as all of the flavin was found in the ultrafiltrate on centrifuging through a Centricon 30 microconcentrator. In a separate experiment the DTT-treated protein was reoxidized by removal of the excess DTT and washing with buffer in a Centricon 30 microconcentrator. This reoxidized sample showed the same 19F resonance as the initial 2',F-arabino-FAD enzyme, consistent with no fluoride elimination being detected in the NMR of the DTT-treated protein, whereas dithionite addition in presence of DTT resulted in the release of fluoride. It should be noted that free 2',F-arabino-FAD and several
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### Table IV
Activities of the Modified Glutathione Reductase Forms

| Assay                                      | NADPH/GSSG<sup>a</sup> | NADPH/TNADP<sup>b</sup> |
|--------------------------------------------|------------------------|-------------------------|
| Native glutathione reductase               | 3,750                  | 1.2                     |
| Arabino-FAD-glutathione reductase          | 300                    | 27                      |
| 2'-F-arabino-FAD-glutathione reductase     | 350                    | 3.5                     |
| 2'-Deoxy-FAD-glutathione reductase         | 2,600                  | <1                     |

<sup>a</sup> Standard assays were performed aerobically in 100 mM sodium Pi, pH 7.6, containing 0.3 mM EDTA, 100 μM each of NADPH and oxidized glutathione at 25°C. The turnover numbers given are in mol of β-NADPH oxidized/min/mol of enzyme. Turnover numbers were calculated from the enzyme-induced rate of β-NADPH oxidation, measured by following the decrease in absorbance of the reduced pyridine nucleotide at 340 nm.

<sup>b</sup> Standard assay was performed in 0.1 M phosphate buffer, pH 7.6, containing 0.3 mM EDTA and 100 μM each of β-NADPH and thio-NADP at 25°C. The turnover numbers are expressed in mol of thio-NADPH formed/min/mol of enzyme. Turnover numbers were calculated from the enzyme-induced rate of thio-NADPH formation, followed by the increase in absorbance at 395 nm.

2'-F-arabino-FAD-reconstituted proteins on reduction with dithionite showed no fluoride elimination over a period of weeks, whereas treatment of the free flavin with DTT resulted in slow fluoride elimination.

Activities of the Reconstituted Glutathione Reductase Forms—Standard aerobic assays were carried out with the reconstituted proteins in 100 mM sodium Pi, pH 7.6, containing 0.3 mM EDTA, at 25°C. The turnover numbers suggest that the activities are hampered in case of arabino- and fluoroflavintreated forms, but with relatively small effect on the 2'-deoxy-FAD-reconstituted form (Table IV). Since the stabilities of the EH2 form of the reconstituted proteins were found to vary, it was thought that the impairment of the activities might be due to the accumulation of the EH2 form during catalysis. This possibility was tested by the NADPH-thionocotinamide NADP transhydrogenase activity of the native as well as the reconstituted proteins (Table IV). The results are in accord with the spectral observations that the EH2 form of the protein was destabilized in case of arabino-FAD-reconstituted glutathione reductase and suggest that the protein may exist significantly in the EH2 form during catalytic turnover. This could result in reduced catalytic activity in the NADPH-GSSG reductase assay, but increased activity in the transhydrogenase assay, which relies only on reduction and oxidation of the flavin. Again rapid reaction studies will be necessary to determine which steps in catalytic turnover are affected by the flavin modifications.

Reconstitution with Apomercuric Reductase—Mercuric reductase is a dimer containing one FAD/monomer and shares many common spectral features with lipoamide dehydrogenase and glutathione reductase. A charge transfer interaction between an active center thiolate and oxidized FAD gives two-electron reduced EH2 a characteristic absorption spectrum as in case of the other two proteins (38). Studies with the 2'-F-arabino-FAD reconstituted mercuric reductase (9, 39) showed that DTT reduces the flavin with no stabilization of the EH2 form, whereas the native protein is stabilized as the two-electron reduced EH2 form when treated with DTT. Hence it was of obvious interest to determine the effect of arabino-FAD as well as 2'-deoxy-FAD on the stability of this catalytically important form of the protein. Apoprotein was reconstituted by incubating with 1.2 eq of modified flavins at 0°C for 60 min. Excess flavin was washed off with 50 mM potassium Pi, pH 7, over a Centricon 30 microconcentrator. The binding of these flavins was accompanied by around 6-8-nm blue shift of the flavin absorption maximum (Table II). Comparatively larger shifts from the free flavin were observed in the spectral region of the near UV peak in the case of the fluororiboflavin-FAD enzyme.

Reduction of Reconstituted Mercuric Reductase Proteins with DTT; Stability of EH2 Form—The arabino-FAD reconstituted mercuric reductase was reduced with excess DTT, and the reduction was followed spectrophotometrically (Fig. 7A). Reduction of the flavin is observed with no detection of the charge transfer band typical of the EH2 form, similar to the results reported for the 2-F-arabino-FAD enzyme (9, 39). When the 2'-deoxy-FAD-reconstituted protein was reduced with DTT, slow but full development of the charge transfer band for the EH2 form was obtained and found to be stable over a period of several hours without any further reduction (Fig. 7B).

19F NMR Studies—19F NMR spectra for the oxidized form of the 2'-F-arabino-FAD reconstituted mercuric reductase showed a signal at 72.3 ppm, which is in the same position as for the other two members of the family, suggesting closely similar chemical environments experienced by the fluorine in all these proteins (Table I).

Activities of the Reconstituted Mercuric Reductase Forms—Standard aerobic assays were carried out with all the reconstituted proteins in 50 mM potassium Pi, pH 7.3, at 37°C (Table V). The turnover numbers suggest that the activities are significantly decreased for all the reconstituted proteins. Reconstitution of lipoamide dehydrogenase and glutathione reductase with 2'-deoxy-FAD resulted in relatively active forms when compared with the arabino-FAD reconstituted forms. In the case of mercuric reductase, although the EH2 form is stabilized, the 2'-deoxy-FAD enzyme is as inactive as the other two forms.

**Conclusions**

The present studies have shown that modification at the 2'-position of the ribityl side chain has a pronounced effect on the catalytic activities of this family of disulfide oxidoreductases. Reconstitution of the proteins with arabino FAD destabilizes the EH2 form and the accumulation of the EH2 form during catalysis is suggested. This concept is supported by the transhydrogenase activities of the glutathione reductases. The de-

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<sup>3</sup> Y. V. S. N. Murthy and V. Massey, unpublished results.
stabilization of the $E_H^2$ form could be attributed to the absence of the 2'-hydroxy group, which stabilizes the thiolate of the $E_H^2$ form in the native enzyme (3). However, it is interesting to note that the deoxy-FAD- and the fluoro-FAD-reconstituted proteins result in relatively stable $E_H^2$ forms. It is possible that the $E_H^2$ form might be stabilized by a solvent molecule through a hydrogen bond, which can take the place of the hydroxy group. During catalysis, the formation of the $E_H^2$ form is excluded in the native $E_H^2$ proteins by the fact that the $E_H^2$ reduction potentials are lower than that for NADPH (35, 10). Although the redox potentials of the free modified flavins are essentially the same as native FAD, the effect of binding at the active site might alter them in such a way that they can influence catalysis. Results obtained from the pyridine nucleotide reduction experiments suggest that NAD$^+$ binding influences the interaction between the flavin and the reduced disulfide in the 2'-arabino-FAD-lipoamide dehydrogenase, presumably by altering the relative oxidation-reduction potentials. It is unlikely, however, that the effects on catalytic activity can be rationalized solely on the thermodynamic stability of the $E_H^2$ forms, since with lipoamide dehydrogenase, all three modified enzymes have much lower activities than native enzyme, despite some of them having thermodynamically stable $E_H^2$ forms. A similar lack of correlation is found with mercuric reductase. Detailed rapid reaction kinetics studies will be required to probe these questions further. The studies have provided $^{19}$F NMR data for different forms of the lipoamide dehydrogenase, which suggest marked conformational changes from one form to the other. The data for the oxidized form of all the three proteins of this family suggest that the fluorine experiences chemically very similar environments at the active site. As anticipated, the 2'-arabinoflavin showed marked stability toward general nucleophiles like sulfide, hydroxide, normal thiols and reduction with sodium dithionite. However, the elimination of the fluorine substituent observed in presence of DTT suggests that the fluorine is susceptible to nucleophilic attack by very reactive reagents.