2′-Fluoro-2′-deoxy-D-arabinoflavin: Characterization of a Novel Flavin and Its Effects on the Formation and Stability of Two-Electron-Reduced Mercuric Ion Reductase†

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ABSTRACT: With the goal of generating a novel fluorine-containing flavin analogue with a reduction potential the same as normal flavin, 2′-fluoro-2′-deoxy-D-arabinoflavin has been synthesized. In its riboflavin and FAD forms, UV−visible spectral properties are similar to those of normal flavins, and tight binding to riboflavin binding protein and mercuric ion reductase occurs with very similar spectral changes. The reduction potential of the 2′-FaFAD analogue is determined to be −207 mV compared with −206 mV for FAD, indicating that the intervening 1′-methylene group insulates the redox-active isoalloxazine from the 2′-fluorine. With the intent of using the analogue as a fluorine NMR probe of the active site environments of two-electron-reduced mercuric ion reductase, apoenzyme was reconstituted and its behavior under reducing conditions examined. Whereas with normal enzyme, addition of two electrons gives rapid formation of a charge-transfer species where FAD remains oxidized and a disulfide is reduced to a thiol/thiolate pair, with the 2′-FaFAD enzyme, addition of two electrons gives rapid reduction of the flavin followed by slow transfer of electrons to the disulfide with very little development of the typical charge-transfer absorption. Analysis of crystal structure data suggests that having the fluorine in the alternate arabino stereochemistry places it much nearer the flavin-proximal cysteine/cystine sulfur, where it may inhibit both electron transfer from reduced flavin and the charge-transfer interaction between reduced thiolate and FAD.

Flavin disulfide oxidoreductases catalyze two-electron transfer reactions between pyridine nucleotide and disulfide/dithiol substrates (Williams, 1992). All members of the class, with the exception of thioredoxin reductase, have homologous homodimeric structures with two interfacial active sites. In each active site, a cystine redox-active disulfide is located perpendicular to the flavin isoalloxazine ring, and the binding site for pyridine nucleotide substrates is located on the opposite face of the isoalloxazine. With this orientation, electrons flow, in the disulfide reductase direction, from pyridine nucleotide through the flavin to the redox-active disulfide to form a two-electron-reduced enzyme denoted EH_2 (see Scheme 1 for mercuric ion reductase).[1] The redox-active thiol/thiolate pair of EH_2 then directly reduces the disulfide substrate, returning the enzyme to the fully oxidized state.

Mercuric ion reductase is a structural homolog to the disulfide reductases (Schiering et al., 1991), but possesses the unique ability to reduce Hg(II) to Hg^0 (Fox & Walsh, 1982). One distinctive feature that contributes to the protein's unusual reactivity is the presence of four cysteine thiols at the active site, the redox-active pair and an additional pair, that participate in liganding interactions with Hg(II) (Moore & Walsh, 1989; Miller et al., 1989). In contrast to the chemistry of the disulfide reductases, the cysteines do not reduce Hg(II) but simply provide a binding site adjacent to the flavin redox mediator (Miller et al., 1986). Reduction of the cysteine-bound Hg(II) requires transfer of electrons from bound NADPH through the flavin. Thus, the pyridine nucleotide complexes of EH_2 are key players in the catalytic mechanism of this enzyme, and understanding their behavior is essential to understanding the catalytic mechanism.

In this vein, we have recently shown, from extensive studies of wild type and site-directed mutant forms of Tn*501*-encoded mercuric ion reductase, that various pyridine nucleotide complexes of the enzyme exhibit asymmetric environments at the active sites (Miller et al., 1991). In our efforts to elucidate both the mechanistic role and structural basis for the asymmetry in this protein, we are exploring the use of alternative probes to directly monitor differences in the active site environments of EH_2 complexes with a variety of ligands. It has previously been shown with other flavoproteins that ^{19}F NMR provides a sensitive probe of changes in redox state and liganding of enzymes reconstituted with the fluorine-containing flavin analogue 8-fluoro-8-demethylflavin (Macheroux et al., 1990). When mercuric ion reductase was reconstituted with this analogue, NMR spectra could be obtained with both liganded and unliganded enzyme in its oxidized state (Miller, 1994). However, the

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[1] Abbreviations: DTT, dithiothreitol; EH_2, two-electron-reduced enzyme; 2′-FaF, 2′-fluoro-2′-deoxy-D-arabinoflavin; 2′-FaFAD, 2′-fluoro-2′-deoxy-D-arabinoflavin adenine dinucleotide; 2′-FaFMN, 2′-fluoro-2′-deoxy-D-arabinoflavin mononucleotide; HPLC, high-performance liquid chromatography; MR-2′-FaFAD, mercuric ion reductase reconstituted with 2′-FaFAD; RBP, riboflavin binding protein; rF, riboflavin; TCA, trichloroacetic acid; TLC, thin layer chromatography.

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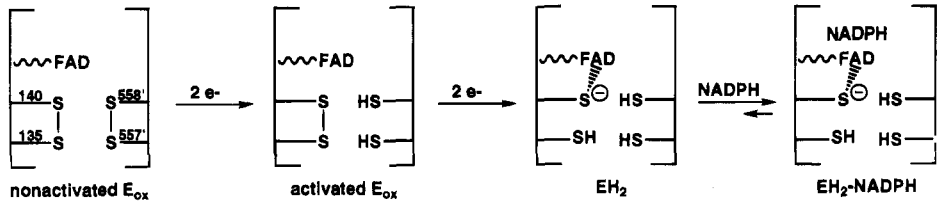
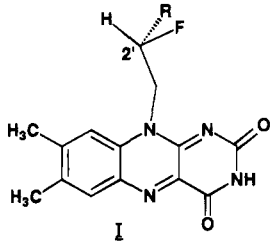
Scheme 1: Active Site Cartoon of Tn*501* Mercuric Ion Reductase Showing Redox States and Pyridine Nucleotide Binding Site[a]



[a] Other disulfide reductases lack the C-terminal cysteine pair.

distribution of electrons at the EH_2 level of the enzyme favored flavin reduction instead of disulfide reduction because the reduction potential of the 8-fluoro analogue is more positive than that of normal flavin. Hence, this analogue is not suitable.

The promising ^{19}F NMR results obtained with oxidized enzyme (Miller, 1994) have inspired us to investigate alternative fluorine-containing flavin analogues with reduction potentials closer to that of FAD. Placement of a fluorine substituent anywhere on the isoalloxazine ring would be expected to raise the reduction potential relative to FAD. However, placement of the fluorine somewhere on the ribityl side chain would insulate it from the ring by one or more methylene groups and should minimize the electronic effects on the reduction potential. Furthermore, since the ribityl side chain is extended up over the redox-active disulfide in the disulfide oxidoreductases (Karplus et al., 1989; Schiering et al., 1991), a fluorine substituent placed there should still be in sufficient proximity to the active site to provide a sensitive probe of the electronic environments of the active sites. As our first derivative, we have synthesized a novel 2′-fluoro-2′-deoxy-D-arabinoflavin (**I**) in which the fluorine substituent



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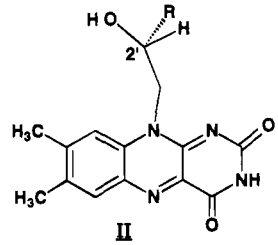

II

is insulated from the redox ring of the flavin by one methylene group. As will be shown below, the analogue has very similar properties to normal flavins in both the riboflavin and FAD forms, including a reduction potential identical to that of normal FAD. It is further shown that apo-mercuric ion reductase binds the FAD analogue tightly with no apparent disruption of pyridine nucleotide binding. However, the presence of the 2′-fluoro group dramatically alters both the kinetics of reduction and the redox equilibria between the flavin and disulfide redox centers in mercuric ion reductase. Analysis of these results in accord with the crystal structure data is presented.

EXPERIMENTAL PROCEDURES

Materials. The C10AC13A double mutant form of Tn*501* mercuric reductase (a wild type equivalent) was purified as previously described (Moore et al., 1992) from *Escherichia coli* strain W3110 *lacI*^{q} harboring plasmid pMMOa10a13 (Moore & Walsh, 1989). Apo-riboflavin binding protein, apo-flavodoxin, and milk xanthine oxidase were kind gifts of Dr. Vincent Massey. 2-Fluoro-2-deoxy-1,3,5-tri-*O*-benzoyl-α-D-arabinofuranose, *Naja naja* venom, and 6-chlorouracil were from Sigma. All other reagents and chemicals were of the highest grade available and were used without further purification.

Methods. All experiments with enzymes were conducted in 50 mM potassium phosphate buffer, pH 7.3, at 4 °C unless indicated otherwise. UV−vis spectra were recorded using a Hewlett-Packard 8452A diode array spectrophotometer. Anaerobic experiments were carried out as previously described (Miller et al., 1991). ^{19}F NMR measurements were performed with a 5 mm ^{19}F probe, on a General Electric GN 500 NMR spectrometer, operating at 470 MHz. Molecular graphics images were produced using the MidasPlus program (Ferrin et al., 1988) at the Computer Graphics Laboratory at the University of California, San Francisco (supported by NIH RR-01081).

Synthesis of 2′-Fluoro-2′-deoxy-D-arabinoflavin (**I**). The following procedures were used to obtain a very low yield (ca. 1%) of the desired material. An optimized procedure for the synthesis will be presented elsewhere (Murthy and Massey, unpublished results). 2-Fluoro-2-deoxy-D-arabinose (**IV**) was obtained by reaction of 2-fluoro-2-deoxy-1,3,5-tri-*O*-benzoyl-α-D-arabinofuranose (**III**) with concentrated NH_4OH in methanol (1:1 solution) overnight at room temperature, followed by evaporation of solvent and column chromatography (silica gel; mobile phase: 97.5:2.5 ethyl acetate/glacial acetic acid). 1-(2′-Fluoro-2′-deoxy-D-arabinotylamino)-3,4-dimethylbenzene (**V**) was obtained by formation and reduction of the Schiff's base (of **IV** and 3,4-dimethylaniline) by procedures described in Ashton et al. (1979). Condensation of **V** with 6-chlorouracil in H_2O according to Ashton et al. (1979), followed by nitrosation (Yoneda et al., 1976), yielded the (N_5)-*N*-oxide of 2′-fluoro-2′-deoxy-D-arabinoflavin (**VI**). Treatment of **VI** with sodium dithionite, followed by H_2O_2 (Yoneda et al., 1976), yielded the desired 2′-fluoro-2′-deoxy-D-arabinoflavin (**I**). The final aqueous reaction mixture was applied to a C_{18} Sep-Pak (Millipore) column, pre-equilibrated with water. The column was washed with water to remove salts and 5% CH_3CN in water to remove more polar impurities, and **I** was then eluted with 10% CH_3CN in water. Fractions having similar UV−visible absorption spectra and similar R_f values on TLC (spots located by fluorescence, silica; mobile phase: 80:20 $CHCl_3$/MeOH) to those of riboflavin were combined and evaporated to dryness.

Conversion of 2′-Fluoro-2′-deoxy-D-arabinoflavin to 2′-Fluoro-2′-deoxy-D-arabinoflavin Adenine Dinucleotide (2′-FaFAD).[1] Conversion of **I** to the FAD analogue was achieved using a crude preparation of the FAD synthetase complex from *Corynebacterium ammoniagenes* (ATCC 6872, previously described as *Brevibacterium*) as described by Spencer et al. (1976). The reaction was followed by TLC as described above (FMN and FAD remain at the origin) or by reverse phase HPLC (C_{18}, 5 × 100 mm; mobile phase:

20% MeOH/80% 5 mM NH_4OAc, pH 6.0, 1 mL/min; retention times: 2′-FaF, 10 min; 2′-FaFMN, 3.7 min; 2′-FaFAD, 2.4 min). When reaction was complete, the solution was centrifuged to remove any precipitated solids, and the supernatant was loaded onto a prewashed Sep-Pak C_{18} column, which was washed first with H_2O, followed by stepwise washes of 3%, 7%, and 10% CH_3CN in water. The water wash contained ADP and small amounts of both 2′-FaFMN and 2′-FaFAD, perhaps bound to Mg^{2+}. The 3% wash contained pure 2′-FaFAD by HPLC, and the higher percentages contained additional small amounts of 2′-FaFMN and 2′-FaF. All samples were evaporated to dryness to remove acetonitrile and redissolved in water before further use.

Apo-Mercuric Ion Reductase Preparation. The apoenzyme of wild type Tn*501* mercuric ion reductase is generated by dialysis at 4 °C against 0.1 M sodium acetate buffer, pH 4.5, containing 5 mM EDTA, 2 M potassium bromide, and a scupula full of activated charcoal. Complete removal of FAD usually requires 2−3 changes of buffer, and the protein typically precipitates in the dialysis bag. When no more fluorescence is observed using a hand-held UV lamp, the buffer is exchanged, and the protein redissolved, by dialysis against the desired buffer, in this case, 50 mM potassium phosphate buffer, pH 7.3, containing 0.3 mM EDTA. If the final enzyme solution retains any characteristic flavin fluorescence or absorbance in the 450 nm region, the procedure is repeated.

Extinction Coefficient Determinations. The extinction coefficient of the purified 2′-FaF was determined by titration with a solution of apo-riboflavin binding protein, which was standardized by titration with pure riboflavin (**II**). The extinction coefficient of the purified 2′-FaFAD was determined by titration with apo-mercuric ion reductase, which was standardized by titration with pure FAD.

Redox Titration of 2′-FaFAD. The redox titration of 2′-FaFAD was performed at pH 7.0 and 25 °C using the xanthine/xanthine oxidase method as previously described (Massey, 1991; Miller et al., 1991). Anthraquinone-2-sulfonate ($E'_0 = -225$ mV; Fultz & Durst, 1982) was used as the redox indicator. Observed potential values were calculated from the Nernst equation using concentrations of oxidized and reduced dye measured by the change in absorbance at the isosbestic point for flavin reduction (336 nm). Concentrations of oxidized and reduced flavin were measured by the change in absorbance at the isosbestic point for dye reduction (354 nm).

Reaction with DTT. Mercuric ion reductase reconstituted with 2′-FaFAD was treated with dithiothreitol to reduce the disulfide that forms between the C-terminal cysteines (557 and 558)[2] upon extended dialysis, as previously described, except the enzyme was maintained at 0−4 °C during incubation (Miller et al., 1989).

[2] In the original description of the Tn*501* gene sequence (Brown et al., 1983), the C-terminal cysteines were numbered as 557 and 558, consistent with the numbering of the N-terminal cysteines as 10 and 13 and the redox-active cysteines as 135 and 140 in the mature protein lacking the amino terminal methionine. Subsequently, in the papers on mutagenesis, the mature numbering of the N-terminal and redox-active cysteines was retained, but the C-terminal cysteines were numbered 558 and 559, inconsistent with the others, but correct for the unprocessed protein (Distefano et al., 1989; Moore & Walsh, 1989; Moore et al., 1992). Here and in future papers, we return to a consistent numbering system for the *mature* protein.

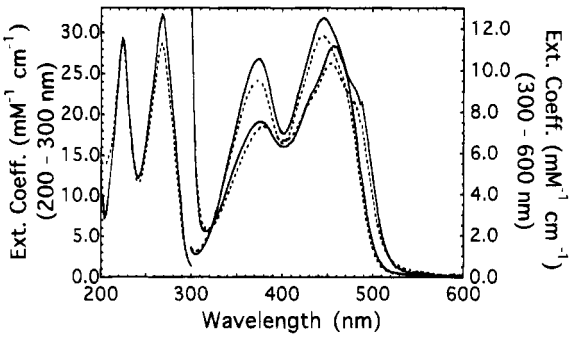

FIGURE 1: Determination of extinction coefficient for 2′-fluoro-2′-deoxy-D-arabinoflavin by reconstitution with apo-riboflavin binding protein (RBP) and comparison with riboflavin. Solid line spectra show riboflavin free (λ_{max} 445 nm) and bound to RBP (λ_{max} 456 nm). Dashed spectra show 2′-fluoro-2′-deoxy-D-arabinoflavin, free (λ_{max} 444 nm) and bound to RBP (λ_{max} 454 nm). In the region 200−300 nm, only the spectra of the free flavins are shown.

TCA Precipitation. Samples (1.0 mL) of unliganded 2′-FaFAD and MR-2′-FaFAD before DTT, after DTT treatment, and after reaction with NADH, all in 50 mM potassium phosphate buffer, pH 7.3, were brought to ca. pH 1 by addition of 100 μL of 50% trichloroacetic acid at 0 °C. After 5 min on ice, the samples were centrifuged at 12500*g* to precipitate protein. A 0.95 mL aliquot of each supernatant was brought to pH 6.5 by addition of 34 μL of 10 N KOH. UV−visible spectra were recorded and HPLC analysis was performed as described above. The precipitated protein samples were washed with water and then redissolved in 1 mL of 8 M guanidine hydrochloride and showed no evidence of any flavin absorbance in the UV−visible spectra.

Enzyme Assays. Assays of mercuric ion reductase reconstituted with 2′-FaFAD contained 100 μM NADPH, 1 mM 2-mercaptoethanol, 50 μM $HgCl_2$, and 100 nM enzyme active sites in 50 mM potassium phosphate buffer, pH 7.3, 25 °C.

RESULTS

Synthesis and Characterization of 2′-Fluoro-2′-deoxy-D-arabinoflavin. The first synthesis of this compound, described here, was not optimized and gave only ca. 1% yield overall (6 μmol); an improved synthesis will be presented elsewhere by Murthy and Massey (unpublished results). In spite of the low yield, the desired material was easily identified as it migrated only slightly ahead of riboflavin on thin layer chromatography (rF: $R_f = 0.4$; 2′-FaF: $R_f = 0.425$) and exhibited a similar greenish fluorescence upon UV irradiation. The material was purified on a Sep-Pak C_{18} column and was eluted under conditions identical to those used to elute riboflavin. As illustrated in Figure 1, the absorption spectrum of 2′-FaF is quite similar to that of riboflavin throughout the 200−600 nm region.

To demonstrate that the isolated material was indeed an analogue of riboflavin and to determine the extinction coefficient, a sample of apo-riboflavin binding protein, standardized the same day by titration with pure riboflavin, was titrated with the purified product. The 2′-FaF product binds stoichiometrically to apo-RBP, yielding similar spectral changes to those that occur upon binding of riboflavin to the protein. As shown in Figure 1, the extinction coefficients determined for 2′-FaF, both on and off the protein, are slightly lower than those for riboflavin (free rF: $\lambda_{max} = 445$ nm, $\epsilon = 12.5$ mM^{-1} cm^{-1}; RBP-rF: $\lambda_{max} = 456$ nm, $\epsilon =$

11.2 mM^{-1} cm^{-1}; free 2′-FaF: λ_{max} = 444 nm, ϵ = 11.7 mM^{-1} cm^{-1}; RBP-2′-FaF: λ_{max} = 454 nm, ϵ = 10.4 mM^{-1} cm^{-1}).

Formation and Characterization of 2′-FaFAD. Since the primary interest in this flavin is as a probe of the active sites of flavin-containing enzymes that bind either the FMN or FAD form of the cofactor, the 2′-FaF was converted to 2′-FaFAD using the FAD synthetase complex from *C. ammoniagenes* (Spencer et al., 1976) before further characterization of its properties. At pH values near 6, the synthetase readily converted the 2′-FaF to a more hydrophilic species as monitored by TLC. However, attempts to reconstitute apo-mercuric ion reductase with the pH 6 product indicated that only 35−40% of the flavin bound to the protein. Since the product had been purified by elution from a Sep-Pak column under conditions that elute both FMN and FAD (5% CH_3CN in water), the sample was tested for the presence of flavin at the FMN level by addition of an FMN-binding protein. Thus, addition of excess apo-flavodoxin to a solution of the 2′-fluoroflavin resulted in decreased extinction in both absorption bands with no change in the λ_{max} at 445 nm but a shift from λ_{max} 372 to 376 nm (data not shown). These changes are typical of the binding of FMN to apo-flavodoxin (Mayhew, 1971). Upon filtration of the flavodoxin solution through a Centricon-10 filter, the filtrate retained ca. 50% of the original flavin absorbance, suggesting that the sample is a 50:50 mix of 2′-FaFMN and 2′-FaFAD.

In addition to binding to apo-flavodoxin, the fluorescence of a sample of the product was measured before and after treatment with *N. naja* venom, which hydrolyzes 2′-FaFAD to 2′-FaFMN. As expected for a mixture of flavins at the FMN and FAD levels, the fluorescence intensity of the sample only increased to 1.5-fold the original value instead of the expected 10-fold typical of pure FAD, again suggesting that only 40−50% of the flavin is present at the FAD level.

Nearly complete conversion of 2′-FaF to 2′-FaFAD (monitored by HPLC) was achieved by raising the pH of the synthetase reaction to 7.0. An improved separation of the 2′-FaFAD from the remaining 2′-FaFMN was achieved by elution of the 2′-FaFAD from the C_{18} Sep-Pak column with 3% CH_3CN in water instead of 5%. The ^{19}F NMR spectrum of the purified 2′-FaFAD (ca. 1 mM in 50 mM potassium phosphate, pH 7.3, containing 10% D_2O) exhibited a single peak with a chemical shift of 66.3 ppm relative to an external hexafluorobenzene standard.

With the fluorine substituent insulated from the isoalloxazine ring system by a methylene group, it is expected that the reduction potential of 2′-FaFAD will be similar if not identical to that of FAD. The reduction potential was determined using the xanthine/xanthine oxidase method of Massey (1991) with anthraquinone-2-sulfonate as the redox indicator. Figure 2 shows spectra for the oxidized and reduced forms of both 2′-FaFAD and anthraquinone-2-sulfonate with isosbestic points at 336 nm for flavin reduction and at 354 nm for dye reduction. A plot of the observed potential *vs* log [ox/red] for 2′-FaFAD (Figure 2, bottom inset) yields a slope of 31 mV, consistent with a simple two-electron process, and a midpoint potential for 2′-FaFAD of E'_0 = −206 mV, which is essentially identical to the value reported for pure FAD (−207 mV: Massey, 1991).

Reconstitution of Apo-Mercuric Ion Reductase and Determination of Extinction Coefficients for 2′-FaFAD. Apo-mercuric ion reductase, standardized by reconstitution with pure FAD, was reconstituted with purified 2′-FaFAD. As was observed for binding of the 2′-FaF to riboflavin binding protein, the 2′-FaFAD binds stoichiometrically to mercuric ion reductase and showed no detectable decrease in absorbance upon ammonium sulfate precipitation and passage through a gel filtration column (Sephadex G-25), indicating very tight binding of the 2′-FaFAD as is seen for pure FAD. As illustrated in Figure 3, the spectral properties of the two flavins, both free and bound to mercuric ion reductase, are quite similar (FAD: λ_{max} = 450 nm, ϵ = 11.3 mM^{-1} cm^{-1}; MR−FAD: λ_{max} = 456 nm, ϵ = 11.3 mM^{-1} cm^{-1}; 2′-FaFAD: λ_{max} = 446 nm, ϵ = 11.5 mM^{-1} cm^{-1}; MR-2′-FaFAD: λ_{max} = 456 nm, ϵ = 11.5 mM^{-1} cm^{-1}). The ^{19}F NMR spectrum of the oxidized MR-2′-FaFAD exhibited a single peak with a chemical shift of 72.3 ppm relative to an external hexafluorobenzene standard, indicating that the

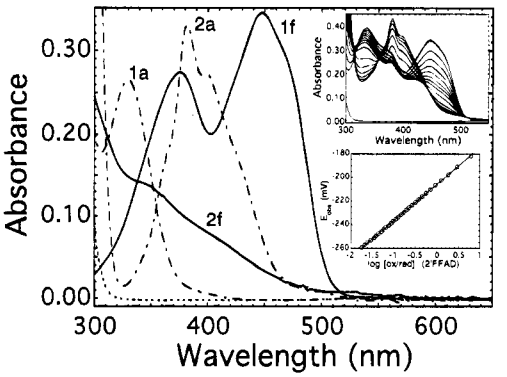

FIGURE 2: Redox characterization of 2′-FaFAD. Spectra of oxidized (1f) and reduced (2f) 2′-FaFAD (29.6 μM) obtained by anaerobic photoreduction in the presence of 3 mM EDTA and 100 mM potassium phosphate, pH 7.0, 4 °C. Also shown are spectra of oxidized (1a) and reduced (2a) 2-anthraquinonesulfonate (50 μM) used as the redox indicator for determination of the redox potential. Dashed spectrum is of 330 mM xanthine. Insets: Top: Anaerobic reduction of 2′-FaFAD (29.6 μM) with xanthine (330 mM) + milk xanthine oxidase (0.0008 A_{450}), in the presence of anthraquinone-2-sulfonate (50 μM), benzyl viologen (1.0 μM), 6 mM EDTA, and 100 mM potassium phosphate, pH 7.0, 25 °C. Spectra with *decreasing* absorbance at 448 and 338 nm are at 0, 0.7, 2.2, 3.7, 5.2, 6.7, 8.2, 9.7, 11.2, 14.2, 21.7, 29.2, 36.7, 44.2, and 51.7 min after addition of xanthine oxidase. Final spectrum (λ_{max} 382 nm) was obtained after photoreduction for 15 s. Bottom: Plot of E_{obs}, calculated from the concentrations of oxidized and reduced dye, *vs* log [ox/red] of 2′-FaFAD. Midpoint potential = −206 mV.

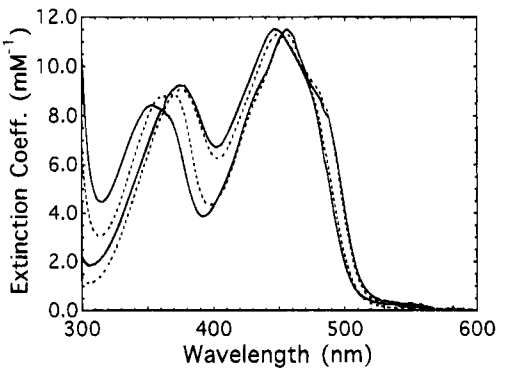

FIGURE 3: Determination of extinction coefficients of 2′-FaFAD by reconstitution with apo-mercuric reductase and comparison with FAD. Solid line spectra show 2′-FaFAD unliganded (λ_{max} 448 nm) and bound to mercuric reductase (λ_{max} 458 nm). Dashed spectra show FAD unliganded (λ_{max} 450 nm) and bound to mercuric reductase (λ_{max} 456 nm).

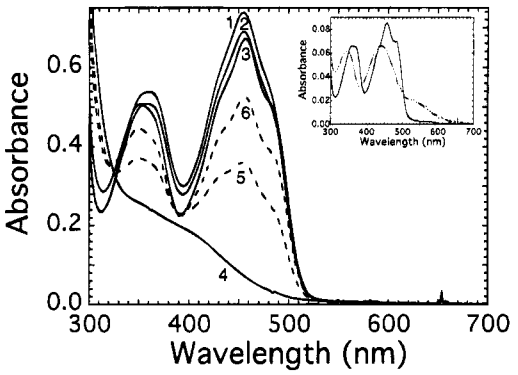

FIGURE 4: Aerobic reaction of 2′-FaFAD-mercuric reductase with dithiothreitol. Solid lines: (1) Oxidized enzyme (64.3 μM); and (2) 10 min, (3) 92 min, and (4) 20 h after addition of 5 mM DTT, maintained at 0−4 °C. Dashed lines: 20 h sample (5) immediately and (6) 30 s after mixing with air. Inset: (—) Oxidized and (— ••• —) two-electron-reduced mercuric ion reductase reconstituted with pure FAD.

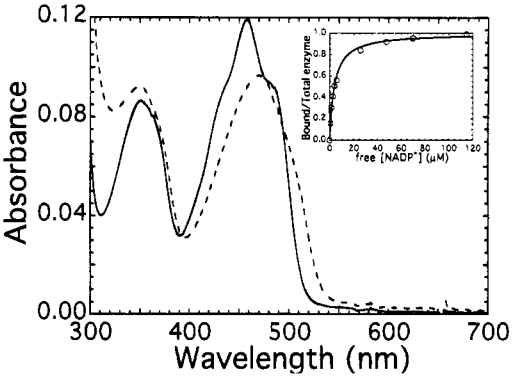

FIGURE 5: Titration of MR-2′-FaFAD with $NADP^+$ in 50 mM potassium phosphate, pH 7.3, 4 °C. (—) Oxidized enzyme (10.3 μM), (- - -) after addition of 232 μM $NADP^+$ (volume corrected). Inset: (O) Fraction of bound enzyme plotted *vs* concentration of unliganded $NADP^+$. Solid line shows a fit of the data to a simple hyperbolic binding curve: fraction bound = $n[NADP^+]/\{K_D + [NADP^+]\}$ with $n = 1$ and $K_D = 3.7 \pm 0.2$ μM. The fit was obtained using the curve fit routine in Kaleidagraph.

fluorine remains intact upon reconstitution of the protein (Murthy & Massey, personal communication).

Reaction with DTT. During the extended dialysis needed to generate apo-mercuric ion reductase, oxidation of the C-terminal cysteines (557 and 558)[2] to a disulfide occurs. Reduction of this disulfide is necessary for full activity of reconstituted enzyme (Miller et al., 1989) and can be conveniently accomplished before or after reconstitution with FAD derivatives by treatment with DTT. In the experiment of Figure 3, apoenzyme was reconstituted immediately after dialysis. In this way, the effect of the 2′-fluoro substituent on the reaction of DTT with the active site disulfides could be followed by monitoring the absorption spectrum of the enzyme-bound flavin. As indicated in Figure 4, addition of DTT at 4 °C leads to a decrease in absorbance in both bands and an enhanced resolution of the shoulders at ca. 430 and 480 nm. After 92 min of incubation (spectrum 3), very little further reaction had occurred, and most importantly, the enzyme showed no development of the typical charge-transfer spectrum that arises during incubation with DTT as the redox-active disulfide also is reduced to a thiol/thiolate pair (Scheme 1 and inset, Figure 4). Hence, incubation with DTT was continued on ice overnight. However, instead of forming the normal charge-transfer species, complete reduction of the flavin was observed (spectrum 4). Upon reoxidation of the flavin with O_2 (spectra 5 and 6), there was still no evidence of the charge-transfer species even though both disulfides are typically reduced by this treatment. The absence of the charge-transfer absorbance could be due to a direct effect of the 2′-F substituent on the interaction between the C140 thiolate and the flavin, or it could occur if DTT or one of the active site cysteines has reacted in a nucleophilic displacement of the 2′-F substituent. To test these possibilities, first the enzyme thiols were quantitated with DTNB after removal of DTT. With normal enzyme, reoxidation of the redox-active cysteines to a disulfide occurs while the C-terminal cysteines remain reduced, and titration with DTNB yields 4 thiols/monomer. With the 2′-FaFAD-reconstituted enzyme, titration with DTNB after removal of DTT gave 4−4.5 thiols/monomer, consistent with the behavior of normal enzyme. To further test for covalent attachment of the flavin to an enzyme cysteine or for reaction with DTT, an aliquot of the enzyme was precipitated with trichloroacetic acid. After centrifugation, the enzyme-free supernatant contained ≥98% of the flavin absorbance, which by HPLC analysis was due to only one species with an identical retention time to that of 2′-FaFAD. These results clearly indicate that the 2′-F substituent remains intact during treatment with DTT. Hence, the absence of charge-transfer absorbance during incubation with DTT suggests that the 2′-F substituent either inhibits reduction of the redox-active disulfide by DTT or significantly perturbs the interaction of the charge-transfer cysteine (C140) with the flavin isoalloxazine ring. This question is examined further below.

Binding of $NADP^+$ to Oxidized MR-2′-FaFAD. After removal of DTT from the reconstituted enzyme, the behavior in binding and redox reactions with pyridine nucleotides was examined. As a test of whether binding interactions are perturbed by the 2′-fluoro substituent, oxidized MR-2′-FaFAD was titrated with $NADP^+$. The spectral changes shown in Figure 5 are very similar to those occurring with normal enzyme (Miller et al., 1991), and the apparent dissociation constant, 3.7 ± 0.2 μM, is also similar to that observed with normal enzyme, 2−4 μM. These results indicate that binding of pyridine nucleotide ligands is not significantly altered by the presence of the 2′-fluoro substituent.

Reaction with NADH. In the incubation with DTT, it is unclear whether the 2′-fluoro substituent prevented reduction of the redox-active disulfide, or simply perturbed the interaction of the resultant "thiolate" with the flavin. To address this question, the anaerobic reduction of the disulfide by NADH via the flavin was examined. With normal enzyme, reaction of NADH with oxidized enzyme (see Scheme 1) gives rapid formation of the two-electron-reduced charge-transfer complex, EH_2, with no evidence for accumulation of any reduced flavin intermediate. Thus, with normal enzyme, transfer of electrons from a putative reduced flavin intermediate to the redox-active disulfide is much faster than transfer of hydride from NADH to the flavin. As shown in Figure 6, reaction of NADH with oxidized MR-2′-FaFAD is quite different. In this experiment, approximately 1 equiv of NADH was tipped into the enzyme from the side arm of an anaerobic cuvette. The first spectrum obtained after the tip (spectrum 2) showed a significant level of flavin reduction but only slight evidence of the typical charge-transfer band at 530 nm (see inset, Figure 4). As

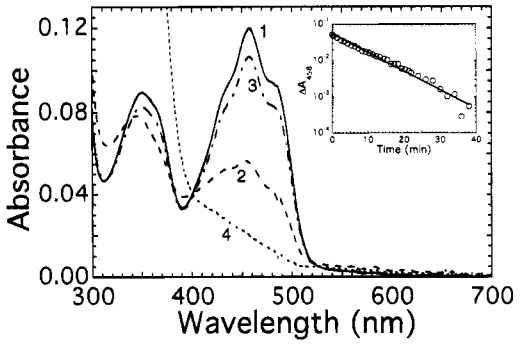

FIGURE 6: Anaerobic reaction of MR-2′-FaFAD with NADH. (1) (—) Oxidized enzyme (10.3 μM); (2) (- - -) 23 s and (3) (— • —) 2 h after addition of 1 equiv of NADH; (4) (•••) after addition of another 5 equiv of NADH. Inset: Log plot of the increase in absorbance at 458 nm after addition of 1 equiv of NADH.

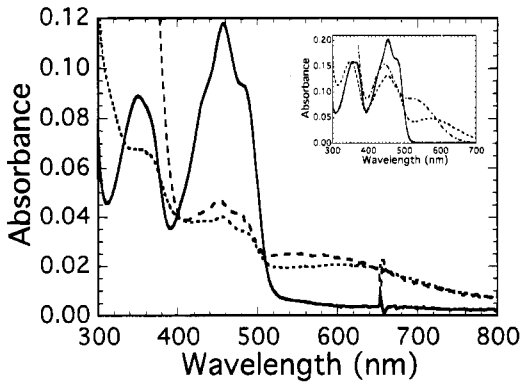

FIGURE 7: Anaerobic reaction of MR-2′-FaFAD with NADPH. (—) Oxidized enzyme (10.3 μM), (•••) +1 equiv of NADPH, (- - -) +6 equiv of NADPH. Inset: Spectra of normal mercuric ion reductase (—) oxidized, (- - -) EH_2—$NADP^+$, (— • —) EH_2—NADPH.

the reaction was monitored, the flavin absorbance returned slowly with a rate constant of $k_{reox} = 0.12$ min^{-1}, but at no time was there any significant development of the typical absorbance at 530 nm (spectrum 3). Further addition of excess NADH resulted in stable reduction of the flavin (spectrum 4), which does not occur with the normal enzyme at pH 7.3. Since the reaction was conducted anaerobically, the return of oxidized flavin absorbance after addition of the first equivalent must be due to transfer of electrons to the redox-active disulfide, but both the rate of electron transfer and the nature of the final reduced cysteines appear to have been significantly perturbed by the presence of the 2′-F substituent. Once again the unusual behavior could arise if an enzyme cysteine reacts to displace the 2′-F substituent; however, TCA precipitation again resulted in ≥98% of the flavin absorbance appearing in the enzyme-free supernatant.

Reaction with NADPH. NADPH and $NADP^+$, the normal substrate and product for mercuric ion reductase, form very tight complexes with two-electron-reduced enzyme (inset, Figure 7). Similar to the reaction with NADH, reaction of normal oxidized enzyme with 1 equiv of NADPH gives rapid formation of the EH_2—$NADP^+$ complex, where the redox-active disulfide is reduced with no evidence for accumulation of reduced flavin intermediates at pH 7.3 (Sahlman et al., 1984). Further addition of NADPH results in ligand exchange, but still no reduction of flavin (inset, Figure 7). As illustrated in Figure 7, the reaction of oxidized MR-2′-FaFAD with NADPH is again quite different from that of normal enzyme. Addition of 1 equiv of NADPH to anaerobic enzyme gives almost complete reduction of the flavin, concomitant with formation of a less intense long wavelength absorbance ($\lambda_{max} \approx 610$ nm) typical of $FADH_2$—$NADP^+$ charge-transfer complexes (Blankenhorn, 1975). Transfer of electrons from reduced flavin to the disulfide appeared to be even slower in this $NADP^+$ complex as only 6—8% of the flavin absorbance returned after ca. 25 min (data not shown) compared to $t_{1/2} \approx 5.8$ min for unliganded enzyme (inset, Figure 6). Addition of excess NADPH yields small spectral changes indicative of exchange of NADPH for $NADP^+$, with formation of an NADPH—FAD charge-transfer interaction (Blankenhorn, 1975). After incubation on ice overnight, ca. 0.25 equiv of NADPH was consumed with very little change in the level of flavin reduction, indicating that additional reduction of disulfide had occurred very slowly. Clearly, the redox behavior of the normal catalytic complexes of EH_2 with NADPH and $NADP^+$ is dramatically perturbed by the presence of the 2′-fluoro substituent on the flavin.

Catalytic Activity of MR-2′-FaFAD. Since Hg(II) binds to the reduced cysteines of EH_2, catalytic reduction of Hg(II) requires electron transfer from NADPH through the flavin. In light of the altered rates of electron transfer to the disulfide observed in the above experiments, it seems likely that transfer of electrons to complexed Hg(II) will also be slower, i.e., the catalytic activity will be significantly lower for the MR-2′-FaFAD enzyme. In aerobic assays at 25 °C, a Hg(II)-stimulated consumption of NADPH was observed, giving a rate of 12.5 min^{-1} compared with a turnover number of 450 min^{-1} for normal enzyme under similar conditions (Moore et al., 1992). The observed rate was the same regardless of whether enzyme was added to initiate reaction or $HgCl_2$ was added to enzyme that had been preincubated with NADPH and 2-mercaptoethanol for up to 15 min. While the NADPH consumption observed under aerobic conditions could result from a Hg(II)-stimulated oxygen reactivity, a slow consumption of NADPH was also observed under anaerobic conditions at 4 °C upon addition of $HgCl_2$ to enzyme premixed with NADPH and 2-mercaptoethanol. Hence, the enzyme does appear to reduce Hg(II), but at a significantly reduced rate.

DISCUSSION

The initial goal of the work presented here was to develop a fluorine-containing flavin analogue suitable for probing the symmetry properties of the EH_2 redox state of mercuric ion reductase homodimers using ^{19}F NMR. Previous work with an 8-fluoro-8-demethylflavin analogue demonstrated that the reduction potential of a suitable fluorine-containing flavin analogue must be equal to or lower than the potential for FAD in order to stabilize the correct electron distribution in EH_2 (Miller, 1994). While placement of the fluorine anywhere on the isoalloxazine ring is expected to raise the potential relative to FAD, placement of the substituent on the ribityl side chain is expected to sufficiently insulate the redox-active ring from the electron withdrawing effects of fluorine that the reduction potential should not be significantly different from that of normal flavin. At the same time, such placement should maintain the substituent within sufficient proximity of the active site for it to be a sensitive probe of redox changes and ligand binding.

In this first attempt to identify a suitable probe, a commercially available 2-fluoro-2-deoxy-D-arabinose deriva-

tive was used to synthesize 2′-fluoro-2′-deoxy-D-arabinoflavin and its FAD analogue. The results presented here demonstrate some success in that the 2′-fluoro substituent effects only minor perturbations on the physical properties of this flavin. Thus, riboflavin and the 2′-FaF analogue are chromatographically similar on both silica gel TLC and reverse phase C_{18} columns; the UV−visible extinction coefficients and λ_{max} values at both the riboflavin and FAD levels of the analogues are nearly identical to those of the normal ribityl derivatives; and most importantly, the reduction potential of the 2′-FaFAD is identical to that of FAD within experimental error.

In terms of protein binding interactions, the results presented here again show little, if any, effect of the 2′-fluoro substituent on the binding of 2′-FaF to riboflavin binding protein or of the 2′-FaFAD dervative to *oxidized* mercuric ion reductase. Changes in extinction coefficients and λ_{max} values upon binding of the 2′-fluoroarabino and normal ribityl derivatives to the proteins are very similar in both cases, indicating that the orientations of the isoalloxazine rings of the analogues in their respective binding pockets are not significantly perturbed from the orientations of the normal ribityl derivatives. Furthermore, while the dissociation constants were not measured explicitly, stoichiometric binding of the appropriate analogue was observed with 10−20 μM concentrations of protein in each case, and no loss of flavin was observed upon gel filtration chromatography, indicating tight-binding behavior. Disruption of a hydrogen bonding interaction to the 2′-hydroxyl group of normal flavin (e.g., 1−2 kcal/mol) by substitution with fluorine might be expected to increase the dissociation constant 10-fold. However, such an increase would not be detected under the conditions used here since the normal protein−flavin dissociation constants are in the nanomolar range. These results clearly indicate that the 2′-fluoro substituent does not dramatically perturb the major binding interactions that serve to orient the flavin in these proteins.

With mercuric ion reductase, the similarity of the spectral changes and the magnitude of the dissociation constant obtained in the titration of E_{ox} with $NADP^+$ further demonstrates that the presence of the 2′-fluoro group does not perturb either the overall binding interactions of the enzyme with pyridine nucleotide or the placement of the nicotinamide ring relative to the isoalloxazine ring of the flavin. In contrast, all of the reactions of E_{ox} with reducing agents are perturbed by the presence of the 2′-fluoro substituent. In normal E_{ox}, the reduction potential of the disulfide is more positive than that of the flavin, so that addition of two electrons yields EH_2 with oxidized flavin and a pair of cysteine thiols (Scheme 1). The redox-active cysteine immediately adjacent to the isoalloxazine ring is stabilized as a thiolate (pK_a ca. 5; Schultz et al., 1985) and participates in a charge-transfer interaction with oxidized flavin, giving rise to the characteristic absorption band at 530 nm (inset, Figure 4). In normal enzyme, formation of this absorption band, indicating reduction of the disulfide, occurs rapidly from either side of the flavin, i.e., by direct reduction of the disulfide with DTT or by transfer of electrons from NADH through the flavin *with no development of a reduced flavin intermediate*. In contrast to the behavior of normal enzyme, reaction of oxidized MR-2′-FaFAD with NADH gave rapid formation of a reduced flavin intermediate, followed by a slow transfer of electrons to the disulfide with very little

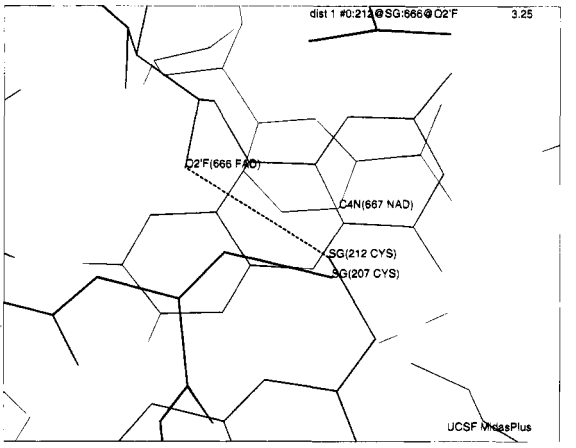

FIGURE 8: Active site of mercuric ion reductase EH_2 from *Bacillus sp.* RC607 with normal FAD and NADPH bound. The charge-transfer cysteine C140 in the Tn*501* enzyme corresponds to C212 in the *Bacillus* enzyme.

absorbance at 530 nm detected. These results indicate that the equilibrium distribution of electrons in 2′-F-EH_2 still favors reduction of the disulfide over the flavin, i.e., that the reduction potential of the disulfide is more positive than that of the flavin. However, the kinetics of electron transfer between the flavin and the disulfide, and the nature of the adjacent cysteine thiol, are significantly perturbed by the 2′-fluoro substituent. The absence of the charge-transfer absorbance at 530 nm indicates that there is no cysteine thiolate interaction with the flavin in EH_2. Disruption of the interaction could be due to protonation of the thiolate, since the thiol does not act as a donor (Miller et al., 1990), or to an increase in the distance between the thiolate and the isoalloxazine ring, since charge-transfer interactions require orbital overlap between donor and acceptor molecules (Mulliken & Person, 1969). Similarly, the decrease in the rate of electron transfer is consistent with either explanation or a combination of both. At pH 7.3, reduction of the disulfide does not normally require protonation of C140, so the protein structure would not be expected to provide a proton donor. However, if the pK_a of C140 is elevated in the presence of the 2′-fluoro substituent, the absence of a proton donor to stabilize the incipient negative charge would be expected to raise the energy of the transition state for reduction. Alternatively, since transfer of electrons from flavin to disulfide appears to occur via formation of a covalent C(4a)-thiol adduct (Miller et al., 1990), an increase in the equilibrium distance between the flavin and disulfide in E_{ox} would be expected to decrease the frequency of encounters between the two and, hence, to result in a decreased rate of electron transfer.

Examination of the active site of mercuric ion reductase from the crystal structure model of enzyme from *Bacillus sp.* RC607 (Schiering et al., 1991) suggests that the 2′-fluoro substituent may cause both an elevation of the pK_a of C140 and a displacement of the disulfide of E_{ox} and the C140 thiol in EH_2 from their normal equilibrium positions. As illustrated in Figure 8, the 2′-hydroxyl group on the normal ribityl side chain lies only 3.2 Å from the C140 thiolate in EH_2. This distance is similar to that seen in the homologous structure of glutathione reductase, where it has been invoked that a hydrogen bond between the 2′-hydroxyl group and the cysteine thiolate, along with a hydrogen bond from a threonine hydroxyl and the interaction of the thiolate with

the flavin π system, all contribute to stabilization of the anionic thiolate in EH_2 (Karplus & Schulz, 1989). However, in the structure of mercuric ion reductase, the hydrogen bonding threonine is replaced by a non-hydrogen bonding valine. In the 2′-FaFAD analogue, two things have changed: (1) the hydroxyl group is replaced by a fluorine atom, which cannot act as a hydrogen bond donor, and (2) the stereochemistry at the 2′-carbon is reversed (see **I** *vs* **II**). Thus, with the analogue bound, not only is there no longer a hydrogen bond donor available to participate in stabilizing the thiolate anion, geometry dictates that the electron-rich fluorine atom must be located closer to the C140 sulfur in both the oxidized and reduced states. Electronic repulsion between the electron clouds of the fluorine and the sulfur could force the disulfide to move farther away from the isoalloxazine ring of the flavin, thereby decreasing the likelihood of covalent interaction between the two needed for efficient electron transfer, as well as eliminating the second stabilizing interaction between the resulting thiolate and the oxidized flavin.

CONCLUSION

While the 2′-FaFAD analogue does not yet satisfy the goal of identifying a fluorine-containing flavin that stabilizes the correct electronic state of EH_2 in mercuric ion reductase, it has provided some initial experimental results consistent with the proposed interaction of the ribityl 2′-hydroxyl group with the thiolate anions in the two-electron-reduced forms of the disulfide oxidoreductase class of enzymes (Karplus & Schulz, 1989). To evaluate the role of the 2′-hydroxyl group more completely, we are currently synthesizing the 2′-fluoro-2′-deoxy-D-**ribo**flavin and 2′-D-arabinoflavin derivatives. These analogues, together with the 2′-deoxy-D-riboflavin, which has recently been used to probe the role of the 2′-hydroxyl group in acyl CoA dehydrogenase enzymes (Ghisla et al., 1994), 2′-FaF, and riboflavin derivatives, will be used to probe both the stereochemical and hydrogen bond donor/acceptor specificity requirements for proper interaction of the 2′-substituent with the active site residues in mercuric ion reductase.

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