^{19}F NMR Studies on 8-Fluoroflavins and 8-Fluoro Flavoproteins†

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ABSTRACT: The ^{19}F NMR spectra of the oxidized and reduced forms of 8-fluororiboflavin, 8-fluoro-FAD, and the 8-fluoroflavin-reconstituted flavoproteins flavodoxin, riboflavin binding protein, D-amino acid oxidase, *p*-hydroxybenzoate hydroxylase, Old Yellow Enzyme, anthranilate hydroxylase, general acyl-CoA dehydrogenase, glucose oxidase, and L-lactate oxidase were measured. For the proteins studied the oxidized resonances appeared over a 10.1-ppm range, while the reduced resonances were spread over 10.3 ppm. Reduction caused an upfield shift of about 27 ppm for the free 8-fluoroflavins and most of the 8-fluoro flavoproteins. The notable exception was 8-fluoro-FMN flavodoxin, which was shifted 37.6 ppm, indicating an unusually high electron density in the benzene ring. Ligand binding to the oxidized 8-fluoro flavoproteins caused either upfield or downfield shifts of 1.5–5 ppm, depending on the protein/ligand combination. The 8-fluoro-FAD anthranilate hydroxylase resonance was shifted downfield and split into two peaks in the presence of anthranilate. The 8-fluoro-FMN Old Yellow Enzyme resonance was shifted upfield upon complexation with charge-transfer-forming, para-substituted phenolates. The upfield shift increased from <1 to 5 ppm as the electron-donating capacity of the phenolate increased. Complexation of native Old Yellow Enzyme with 2,4-difluorophenol caused the fluorine resonances of the ligand to shift and split into two pairs of signals. Each pair of signals was associated with a different isozyme of Old Yellow Enzyme.

Derivatized flavins have been widely used to probe the flavin binding site of flavoproteins [for a review, see Ghisla and Massey (1986)]. Flavins, chemically modified at positions 2 (Claiborne et al., 1982), 4 (Biemann et al., 1984), 6 (Massey et al., 1986a,b), and 8 (Massey et al., 1979; Schopfer et al., 1981) were used in recent investigations to examine the environment provided by the protein and to test the accessibility of these positions to solvent-borne reagents. Müller and coworkers (Van Schagen & Müller, 1981; Vervoort et al., 1986) have taken another approach, studying protein–flavin interactions with flavoproteins reconstituted with ^{13}C-enriched (in position 2, 4, 4a, and 10a) and ^{15}N-enriched (in position 1, 3, 5, and 10) flavins. ^{13}C and ^{15}N NMR spectra were obtained, and the chemical shifts of the carbon and nitrogen ring atoms were compared with the chemical shifts of free flavin. We have performed ^{19}F NMR investigations with flavoproteins reconstituted with 8-fluoroflavins. Miura et al. (1983) first described this approach in a study on riboflavin binding protein reconstituted with 8-fluororiboflavin. 8-Fluoroflavin is a very suitable NMR probe for the following reasons (Gerig, 1978; Emsley & Phillips, 1971): (i) the high sensitivity of fluorine NMR reduces the amount of protein required by 20-fold when compared to ^{13}C NMR and 50-fold when compared to ^{15}N NMR; (ii) the fluorine nucleus is very sensitive to changes in both the environment (through space) and electron density (through bond) and in this respect serves as an adjunct to ^{13}C and ^{15}N NMR; (iii) the steric requirements of the fluorine atom are similar to those of hydrogen; therefore, the 8-fluoroflavin should be easily accommodated in flavoproteins. One potential disadvantage is the electron-withdrawing capacity of a fluorine substituent.

In this paper, we present a survey of the fluorine NMR spectra for both the oxidized and reduced states of 8-fluoroflavin free and bound to a variety of apoflavoproteins. The utility of the method for detecting flavoprotein subpopulations and for characterizing charge-transfer interactions is demonstrated.

Experimental Procedures

Materials

8-Fluororiboflavin was a generous gift from Dr. K. Matsui, Osaka City University. Conversion to the FAD level was achieved by means of the FAD synthetase complex from *Brevibacterium ammoniagenes* as described by Spencer et al. (1976). 8-Fluoro-FMN was prepared from 8-fluoro-FAD with the phosphodiesterase activity of *Naja naja* venom (Sigma).

Preparation of Flavoproteins and Their Corresponding Apoproteins. The holoproteins and apoproteins were prepared as previously described: flavodoxin from *Megasphera elsdenii* (Mayhew & Massey, 1969; Mayhew, 1971); riboflavin binding protein from hen egg white (Becvar & Palmer, 1982); L-lactate oxidase from *Mycobacterium smegmatis* (Sullivan et al., 1977; Choong et al., 1975); Old Yellow Enzyme (Abramowitz & Massey, 1976a,b); D-amino acid oxidase from pig kidneys (Fitzpatrick & Massey, 1982; Massey & Curti, 1966); glucose oxidase from *Aspergillus niger* (Swoboda & Massey, 1965; Mayer & Thorpe, 1981); anthranilate hydroxylase from *Trichosporon cutaneum* (Powlowski et al., 1987; Mayer & Thorpe, 1981); *p*-hydroxybenzoate hydroxylase from *Pseudomonas putida* (Müller et al., 1979; Entsch et al., 1980). General acyl-CoA dehydrogenase from pig kidneys was a generous gift from Dr. C. Thorpe, University of Delaware, and the apoprotein was prepared following the procedure of Mayer and Thorpe (1981).

D(+)-Glucose and L-lactate (lithium salt, optically pure) were from ICN. D-Alanine (A grade) was from Calbiochem. Acetoacetyl-CoA, NADPH (type III), and deuterium oxide (100%) were obtained from Sigma. *p*-Fluorophenol, 2,4-difluorophenol, *p*-nitrophenol, *p*-cresol, *p*-chlorophenol, *p*-

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methoxyphenol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate, and tetradeuteriomethanol (CD_3OD, 99.8%, in sealed ampules) were from Aldrich. Sodium oxalate was from Allied Chemical. Anthranilic acid was from Nutritional Biochemical Co. and was recrystallized three times from water before use. Platinum oxide (81.7%) was from Matthey Bishop, Inc. Sodium dithionite was a generous gift from the Virginia Chemical Co. All other chemicals were of reagent grade and were used without additional purification.

Methods

Apoprotein Reconstitution. Reconstitution of the apoproteins with 8-fluoroflavin was accomplished by mixing an approximately 1.5-fold molar excess of flavin with apoprotein and then incubating on ice (from 30 min to overnight, depending on the protein). Excess flavin was removed either by gel filtration over G-25 (Pharmacia) or in a Centricon microconcentrator (Amicon). Unless otherwise indicated, all samples were prepared in 0.1 M potassium phosphate buffer, pH 7.0.

Extinction Coefficient Determination. The extinction coefficients for free 8-fluororiboflavin and 8-fluoro-FMN were determined by titration with standardized preparations of apo-riboflavin binding protein and apoflavodoxin, respectively (in 0.1 M potassium phosphate buffer, pH 7.0, 25 °C). The same value was obtained for each flavin, 12 500 M^{-1} cm^{-1} at 435 nm, which is significantly higher than the reported value of 10 500 M^{-1} cm^{-1} (Kasai et al., 1983). The value was further confirmed by reacting 8-fluororiboflavin with *N*-acetylcysteine to generate 8-(*N*-acetylcysteinyl)riboflavin, which has an independently established extinction coefficient (Moore et al., 1979). The extinction coefficient for 8-fluoro-FAD (11 700 M^{-1} cm^{-1} at 440 nm) was obtained by comparison with 8-fluoro-FMN after reaction of a pure preparation of 8-fluoro-FAD with *Naja naja* venom phosphodiesterase.

NMR Sample Preparation. Protein samples for the NMR were concentrated by means of a Centricon microconcentrator, yielding 50–150 μL, which was then diluted with an appropriate volume of buffer and 50 μL of D_2O (100% Sigma) to give a final volume of 400 μL. When the sample had been titrated with a ligand, dilution was made from the solution that passed through the Centricon membrane. Thus the concentration of the ligand was unchanged. All protein concentrations refer to the protein-bound flavin (unless otherwise indicated) and were determined spectrophotometrically.

Aqueous reduced samples were prepared by addition of an excess of the reducing agent to an aerobic sample in the NMR tube. The air space over the oxidized sample was first flushed with oxygen-free argon; then, 20–50 μL of the reductant, in buffer, was introduced with a long-needle Hamilton syringe. The final reductant concentration was 2.5 mM in all cases. Argon flushing was continued during and after the addition. The tube was then closed with a standard plastic cap. The sample became bleached within a few minutes and remained reduced for 12–24 h. All operations with reduced samples were carried out under dim light in order to prevent photoelimination of the fluorine from position 8 (Massey et al., 1980). Catalase (1 μL; A_{402} = 3.4) was added prior to reduction in order to prevent H_2O_2, generated during reduction of residual oxygen, from damaging the protein.

Enzymes that were reduced with their normal substrates include 8-fluoro-FAD D-amino acid oxidase with D-alanine, 8-fluoro-FAD *p*-hydroxybenzoate hydroxylase and 8-fluoro-FAD anthranilate hydroxylase with NADPH, 8-fluoro-FAD glucose oxidase with D-glucose, and 8-fluoro-FMN L-lactate oxidase with L-lactate. Sodium dithionite was used to reduce 8-fluororiboflavin, 8-fluoro-FAD, 8-fluoro-FMN flavodoxin, 8-fluoro-FAD general acyl-CoA dehydrogenase, 8-fluoro-riboflavin riboflavin binding protein, and 8-fluoro-FMN Old Yellow Enzyme. Except for the latter two proteins, whenever we used dithionite as a reductant, we noticed the appearance of some free fluoride (43.4 ppm) in the NMR spectrum. In addition, 8-fluoro-FAD *p*-hydroxybenzoate hydroxylase (plus or minus *p*-hydroxybenzoate) released free fluoride when reduced with dithionite but not when reduced with NADPH. Control experiments showed that sulfite, an oxidation product of dithionite, could displace fluoride from oxidized 8-fluoro-riboflavin (0.1 M potassium phosphate buffer, pH 7.0, 25 °C) at a second-order rate of 12 M^{-1} min^{-1}. Reaction of sulfite with reduced 8-fluororiboflavin was much slower, possibly nonexistent.

8-Fluororiboflavin in 100% methanol (CD_3OD) was reduced catalytically, in an Omnifit NMR sample tube system (Wilmad Glass Co.), by gently bubbling the sample with hydrogen gas in the presence of a few grains of platinum oxide. Reduction was complete in 1–2 min. The sample was then sealed under hydrogen.

Ligand Binding. Binding of ligands to selected 8-fluoroflavin-reconstituted enzymes was followed by the changes in the UV–visible absorbance spectrum of the protein-bound flavin. Though not described in detail, titrations monitored in this way permitted the calculation of the various dissociation constants reported. Either a Varian Model 219 double-beam scanning spectrophotometer or a Hewlett-Packard Model HP 8452A diode array spectrophotometer was employed for these measurements. The sample compartments in both instruments were temperature controlled.

NMR Acquisition Parameters. ^{19}F NMR measurements were performed with a 5-mm ^{19}F probe, on a General Electric GN 500 NMR, operating at 470 MHz. The repetition time was 2–3 s with a 12-μs pulse (60° flip angle). All protein NMR measurements were made without broad-band proton decoupling, at 25 °C (Jardetzky & Roberts, 1981). Fluorine chemical shifts are given in ppm from hexafluorobenzene, used as an external standard. Spectra were recorded with 8K or 16K data points over spectral widths from 19 to 44 kHz.

NMR Processing. The base line of an NMR spectrum with broad lines and low signal to noise taken over a wide spectral width is not flat (Fukushima & Roeder, 1981; Lindon & Ferrige, 1980), as was the case in our studies. Therefore, correction routines (GEM routines IC, FB, and BF) were used to flatten the base line. Exponential line broadening was performed as described in the figure legends. Line-width analyses assumed a Lorentzian line shape as did curve analysis and deconvolution.

Results and Discussion

^{19}F NMR of Free 8-Fluoroflavins

Oxidized 8-Fluoroflavins. A single peak was found for oxidized 8-fluororiboflavin at 64.9 ppm (16.6 ± 0.8 Hz); see Figure 1 and Table I. The concentration of free flavin was kept at 50 μM in order to avoid potential complications from dimerization (Gibson et al., 1962; Drabent & Grajek, 1983), though there was no effect on the peak position upon an increase of the concentration to 130 μM. There is a 21.3-ppm difference in chemical shift between 8-fluororiboflavin and fluoride (Table I). This is 2.7 ppm smaller than the difference reported by Miura et al. (1983) in D_2O versus H_2O buffer. Repeating the D_2O experiments, we found the resonance of 8-fluororiboflavin (1.7 mM) was unshifted in the D_2O buffer (65.0 ppm), while the signal for fluoride (10 mM) moved 2.8

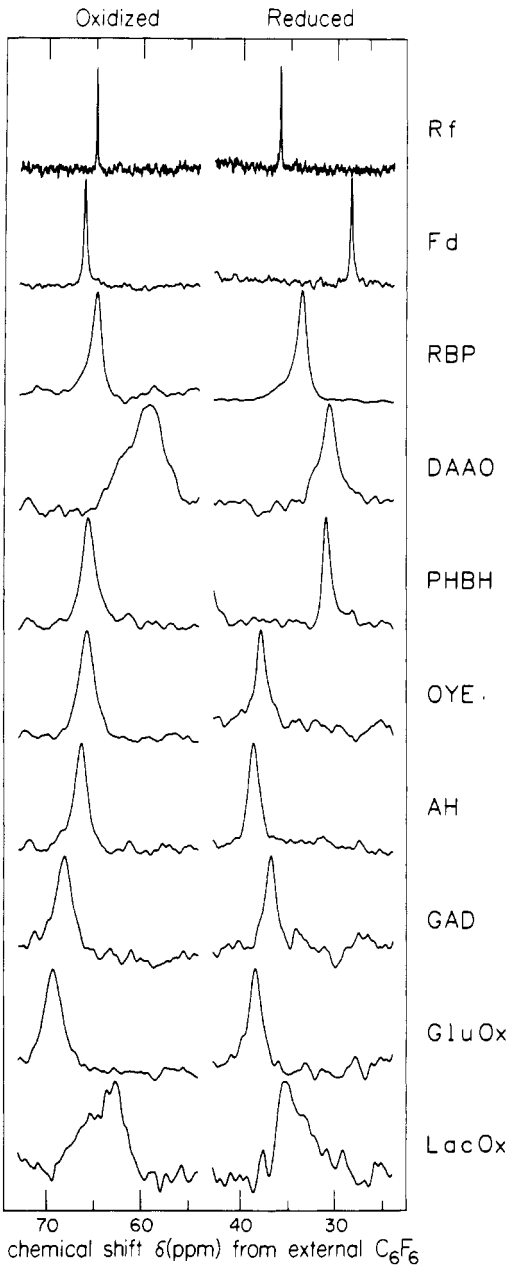
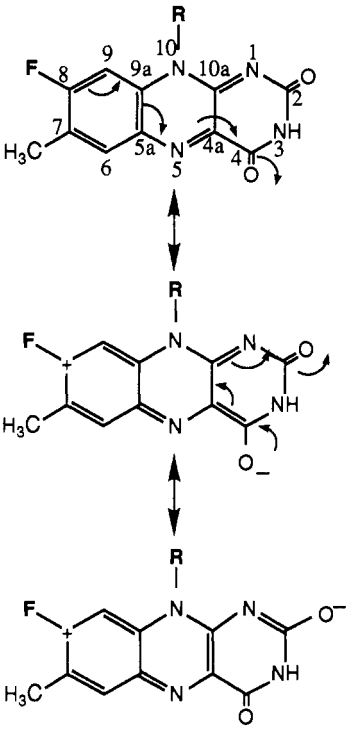


FIGURE 1: ^{19}F NMR spectra of oxidized and reduced 8-fluororiboflavin and 8-fluoroflavin-reconstituted flavoproteins. Conditions: Oxidized samples (with the exception of 8-fluoro-FAD D-amino acid oxidase) were prepared in 0.1 M potassium phosphate buffer, pH 7.0, containing 7% D_2O. 8-Fluoro-FAD D-amino acid oxidase was prepared in 20 mM sodium pyrophosphate buffer, pH 8.5, containing 7% D_2O. The concentration of 8-fluororiboflavin was 50 μM, while that of the 8-fluoroflavoproteins varied between 140 and 450 μM (concentrations for individual samples are given in Table I). The left column shows the oxidized spectra; the right column, the reduced spectra. All spectra were processed with an exponential line broadening (EM) of 300 Hz except reduced and oxidized 8-fluororiboflavin (EM = 30 Hz) and reduced and oxidized 8-fluoro-FMN flavodoxin (EM = 100 Hz). The abbreviations listed on the vertical axis are defined in Table I (footnote *b*). Spectra are presented from top to bottom in the order of increasing molecular weight (except for DAAO; see footnote *c* in Table I).

ppm upfield (to 40.6 ppm), accounting for the difference.

Dissolving oxidized 8-fluororiboflavin in 100% methanol resulted in an upfield shift of the peak to 60.0 ppm. The majority of this shift is attributable to two factors. The first is a change in the bulk magnetic susceptibility of the medium (Emsley & Phillips, 1971). The second is a decrease in the hydrogen-bonding interactions around the pyrimidine moiety of the flavin. The latter effect can be explained in the following

Scheme I: Resonance Structures for the Isoalloxazine Ring System and the Ring Atom Numbering Scheme



way. Hydrogen bonding to the pyrimidine oxygens [C2(=O) and C4(=O)] would be expected to shift electron density from the benzene ring to the pyrimidine ring of the isoalloxazine, thereby deshielding the fluorine, resulting in a downfield shift. Some of the resonance structures that could contribute toward giving the benzene ring more positive character are illustrated in Scheme I. Disrupting the hydrogen bonding would have the opposite effect, increasing electron density in the vicinity of the fluorine, causing an upfield shift. Partial disruption of hydrogen bonding in methanol is indicated by partial resolution of the absorbance spectrum in this solvent (Harbury et al., 1959; Kotaki et al., 1970).

The ^{19}F NMR resonance of oxidized 8-fluoro-FAD in buffer is shifted 0.7 ppm downfield from that of 8-fluororiboflavin to 65.6 ppm (Table I). This can be attributed to the influence of the adenine moiety stacking on the isoalloxazine (Weber, 1950; Miles & Urry, 1968).

Reduced 8-Fluoroflavins. Reduction of 8-fluororiboflavin in aqueous solution caused a large upfield shift to 36.0 ppm (Figure 1 and Table I). Such a shift is consistent with increased electron density near the fluorine. Reduction of 8-fluororiboflavin in 100% methanol caused an upfield shift, to 33.3 ppm.

Both reduced and oxidized 8-fluororiboflavin (Figure 2) are shifted upfield in 100% methanol relative to aqueous buffer. However, the reduced flavin is shifted 2.2 ppm less. In reduced flavin, communication between the pyrimidine and benzene rings (cf. Scheme I) is diminished, due to the sp^3 hybridization of the nitrogens at positions 5 and 10 (Moonen et al., 1984), which restricts the transfer of resonance effects between the rings. When the solvent is changed to methanol, hydrogen bonding is diminished for both the oxidized and reduced states, but in reduced 8-fluororiboflavin, the hydrogen-bonding interactions in the pyrimidine ring cannot affect the electron density at the 8-position. Thus, the fluorine in oxidized 8-fluoroflavin experiences both a change in hydrogen bonding and a change in the bulk magnetic susceptibility of the solvent, while the fluorine in reduced 8-fluoroflavin experiences only

Table I: ^{19}F NMR and Absorbance Parameters of Fluoride, 8-Fluoroflavins, and 8-Fluoro Flavoproteins[a]

			NMR						absorbance oxidized maximum (nm) [extinction (mM^{-1} cm^{-1})][g]		
			oxidized			reduced					
compd[b]	mass (kDa)[c]	concn (μM)[d]	peak (ppm)	width (Hz)[e]	no. of scans[f]	peak (ppm)	width (Hz)[e]	no. of scans[f]			
fluoride	0.019	5000	43.4		28						
D_2O[h]		10000	40.6	12.5 ± 0.3	64						
FAD	0.83	50	65.6	15.8 ± 1.4	13310	35.7	23.8 ± 1.2	5076	356 [7.4]	440 [11.7]	
Rf	0.38	50	64.9	16.6 ± 0.8	12972	36.0	16.6 ± 0.9	6552	355 [7.8]	435 [12.5]	
pH 5.5		50				36.9	19.5 ± 0.8	5164			
pH 8.5		50				36.0	17.6 ± 0.8	8420			
methanol		200	60.0	16.4 ± 0.3	528	33.3	54.0 ± 1.0	5440			
D_2O[h]		1660	65.0	11.7 ± 1.0	604						
Fd	15.1	140	66.1	72 ± 0.7	7664	28.5	28 ± 0.8	5999	355 [9.5]	436 [10.8]	
RBP	34.0	450	64.7	227 ± 4.1	3420	33.7	259 ± 6.9	3236	355 [5.7]	449 [10.5]	
DAAO[i]	39.6	265	59.3	1610 ± 32	60816	30.9	348 ± 14	11996	344 ["9.3"]	438 ["11.7"]	
+benzoate		265	62.5	437 ± 9.6	65536					455 ["12.6"]	480 (sh)[k]
PHBH	88	110	65.8	470 ± 6.5	35644	32.3	447 ± 13	22048	358 ["7.3"]	440 ["11.7"]	
+p-OH-benzoate		110	67.4	849 ± 12	35100	31.2	120 ± 4.2	5832			
OYE	96		65.7	705 ± 6.7	30112	38.1	216 ± 8.6	6628	353 [10.8]	454 [11.5]	
+p-NO_2-phenol			65.8/ 65.0		16887						544 [3.3]
+p-COOH-phenol			64.3/ 62.8		37428				348	466	620
+p-CHO-phenol			64.4/ 63.7		38863				344	422	596
+p-Cl-phenol			61.5		11764				346	440	664
+p-CH_3-phenol			60.5		32800				348	446	674
+p-OCH_3-phenol			60.6		36676					440	716 [4.4]
AH	100	190	66.5	482 ± 12	26312	38.8	370 ± 5.0	12432	352 ["6.7"]	448 ["12.0"]	472 (sh)[k]
+anthranilate		190	69.6/ 67.4		23240	37.3	116	5464		444 ["12.7"]	
+salicylate		110	66.9	331 ± 11	36820						
GAD	164		68.1	656 ± 9.7	28005	36.9	247 ± 7.8	12396	350 ["9.0"]	435 ["15.9"]	
+acetoacetyl-CoA			66.5	1299 ± 26	26132					428	548
GluOx	186	139	69.4	655 ± 9.5	18280	38.5	437 ± 10	13376	355 [7.5]	438 [12.1]	
LacOx[j]	350		63.7	1300 ± 33	20304	35.7	543 ± 16	16384	346 ["6.8"]	448 ["10.6"]	
+phosphate			62.2	459 ± 24	29764	35.1	802 ± 30	17956	362 ["6.8"]	446 ["12.2"]	
+oxalate			62.4	915 ± 23	17508						

[a] Conditions were 0.1 M KP_i buffer, pH 7.0, 25 °C, unless otherwise indicated. [b] Protein abbreviations: Fd, flavodoxin; RBP, riboflavin binding protein; DAAO, D-amino acid oxidase; PHBH, *p*-hydroxybenzoate hydroxylase; OYE, old yellow enzyme; AH, anthranilate hydroxylase; GAD, general acyl-CoA dehydrogenase; GluOx, glucose oxidase; LacOx, L-lactate oxidase. [c] Molecular masses are given for the multimeric form of each protein as found in solution except for D-amino acid oxidase, where the value of the monomer is given. [d] Those samples for which no specific concentration is given were measured at concentrations between 140 and 450 μM. [e] Line widths were measured at half-height, $\Delta\nu_{1/2}$, and corrected for added line broadening. [f] This column records the number of accumulated scans which were averaged to generate the final NMR spectrum. [g] Values enclosed in quotation marks indicate extinction coefficients estimated by comparison with the effect of binding FAD or FMN to native enzyme. Values without quotation marks were measured directly from spectral changes detected on titrating free 8-fluoroflavin with the apoenzyme (data not shown). [h] Measurements were made in 0.1 M sodium phosphate in D_2O adjusted to pH* 7.0. [i] D-Amino acid oxidase measurements were made in 20 mM sodium pyrophosphate buffer, pH 8.5, 25 °C. [j] Lactate oxidase measurements were made in 10 mM imidazole buffer, pH 7.0, 25 °C. When phosphate was present, it was at a concentration of 0.1 M; oxalate was added to a concentration of 6 mM. [k] The sh designation indicates a prominent shoulder.

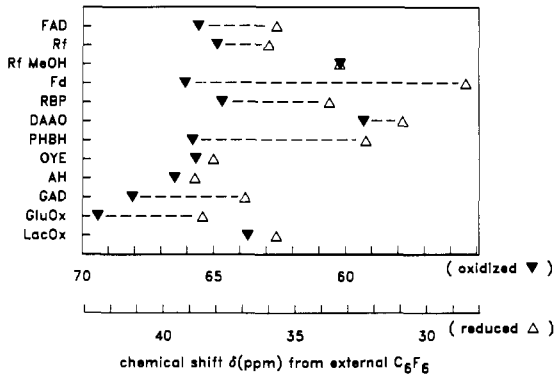


FIGURE 2: Correlation diagram of the relative ^{19}F NMR peak positions from the spectra of oxidized and reduced 8-fluoroflavins and flavoproteins. Conditions: Same as in Figure 1. The oxidized (55–70 ppm) and reduced (28–43 ppm) chemical shift regions have been overlapped by aligning the peak positions for oxidized and reduced 8-fluororiboflavin in 100% methanol. Filled inverted triangles indicate oxidized peak positions; open triangles indicate reduced positions. Abbreviations along the vertical axis are defined in Table I (footnote *b*).

the change in bulk magnetic susceptibility, making the upfield shift less in the reduced state. The 2.2-ppm difference between the reduced and oxidized solvent-dependent shift can be attributed to the effect of hydrogen bonding, while the larger, 2.7 ppm, shift observed with both oxidized and reduced flavin can be attributed to the bulk effect of solvent.

^{19}F NMR of 8-Fluoro Flavoproteins

Stability of 8-Fluoro Flavoproteins. The fluoro derivative of the flavin is more susceptible to nucleophilic attack than is the more thoroughly studied chloro derivative (Kasai et al., 1983). As a preliminary control study, we examined the stability of a number of oxidized 8-fluoroflavin-reconstituted enzymes at pH 7.5, 8.5, and 9.5. Reaction of 8-fluoroflavin with those nucleophiles normally associated with proteins (sulfur, oxygen, or nitrogen) causes large changes in the absorbance spectrum (Moore et al., 1979; Kasai et al., 1983). We found that the proteins used in this NMR study were stable, showing only a few percent reaction over several days.

Number of Peaks. Three fundamental pieces of information can be taken from the ^{19}F NMR measurements on flavoproteins reconstituted with 8-fluoroflavins. First, the number of peaks provides an indication of the number of different flavin-binding environments present in the sample. From Figure 1, it can be seen that the proteins in this study all exhibit single peaks in both their oxidized and their reduced states. As will be described later, in some cases two peaks can be induced upon ligand binding.

Line Width. Second, the ^{19}F NMR signal line width of the protein-bound 8-fluoroflavin can be related to the molecular weight of the protein and is a good indication of protein multimer complex formation. Provided that the 8-fluoroflavin is rigidly bound to the protein, it will exhibit the same tumbling rate (rotational correlation time, τ_c) as the protein. Line widths of NMR signals increase as the τ_c values increase, and τ_c increases as the molecular weight of the protein (or protein multimer) increases (Jardetzky & Roberts, 1981). In addition to the common dipole–dipole relaxation mechanism there is a large contribution, in ^{19}F NMR, to the observed line width from chemical shift anisotropy (CSA) relaxation (Hull & Sykes, 1975). The relative contribution of the CSA mechanism is increased as a result of the high field at which the spectra in this study were recorded. Our results show the general correlation of increasing line width with increasing molecular weight.

An exception to this general tendency is oxidized 8-fluoro-FAD D-amino acid oxidase, which exhibits a much larger line width than predicted for its molecular weight. This finding is however consistent with the well-known tendency for this enzyme to form polymers (Charlwood et al., 1961; Antonini et al., 1966). The line width for reduced 8-fluoro-FAD D-amino acid oxidase is much narrower, suggesting that upon reduction the polymeric enzyme dissociates into smaller aggregates. It is unlikely that the narrowing of the line width is due to release of reduced flavin from the protein because the reduced flavin is bound to the protein orders of magnitude more tightly than oxidized flavin (Brunori et al., 1971; Van den Berghe-Snorek & Stankovich, 1985).

The data in Table I indicate that in general the signal line width for the reduced protein is equal to or narrower than that for the oxidized form. Similar observations have been reported in ^{13}C NMR studies on Old Yellow Enzyme containing ^{13}C-enriched flavin (Beinert et al., 1985b). The line width of free, reduced 8-fluororiboflavin was the same at both pH 5.5 (19.5 ± 0.8 Hz) and pH 8.5 (17.6 ± 0.8 Hz) (Table I). We determined the pK_a of reduced 8-fluororiboflavin to be about 6.5 (data not shown). Therefore, ionization of the reduced 8-fluororiboflavin does not alter its line width.

Peak Position. Third, the position of the NMR peak reflects the environment of the 8-fluoroflavin. We have found peaks spread over a 10.1-ppm range for the oxidized state and a 10.3-ppm range for the reduced state (Table I). This falls within the range of ^{19}F NMR chemical shifts of approximately 12 ppm noted by Gerig (1978) for protein-induced shifts.

By comparing both the oxidized and reduced forms of each 8-fluoro flavoprotein, we have sought to take advantage of the acknowledged environmental sensitivity of the fluorine nucleus while circumventing the difficulties inherent in the interpretation of the chemical shift in terms of defined physical interactions. We have chosen 8-fluororiboflavin in 100% methanol as the reference for aligning the oxidized and reduced regions since hydrogen bonding to the flavin is minimized in 100% methanol. When normalized in this manner, the reduced peak was still shifted upfield relative to the oxidized peak in all cases (Figure 2). For most of the proteins, the difference amounted to only a few ppm, suggesting that there are no major changes in the flavin environment upon reduction. However, two proteins, 8-fluoro-FAD *p*-hydroxybenzoate hydroxylase and 8-fluoro-FMN flavodoxin, showed large, relative upfield shifts for the reduced peak compared to the oxidized peak.

In the flavodoxin case, comparison of the X-ray structures of the oxidized and reduced forms of the closely related flavodoxin from *Clostridium M.P.* shows a single, major protein change on reduction. A backbone carbonyl residue is inverted so as to form a strong hydrogen bond to the proton at N(5) of the reduced flavin (Mayhew & Ludwig, 1975; Ludwig et al., 1975). Except for a minor change in the angle between the indole ring of tryptophan-90 and the flavin plane, there are no other obvious changes in the positions of the residues near the flavin which could account for the 10.6-ppm upfield shift. Though it is possible that there could be a difference in the flavin–protein interactions of 8-fluoro-FMN flavodoxin compared to native flavodoxin or that the structural changes which flavodoxin undergoes on reduction are substantially greater in solution than they appear to be in the crystal, it seems more likely that the NMR shift is caused by the new, strong hydrogen bond at position N(5). This strong hydrogen bond should result in polarization of the benzene ring with increased electron density at the 8-position, consistent with the large upfield shift observed.

The magnitude of the ^{19}F NMR shift on reduction of flavodoxin (37.6 ppm) is emphasized by comparison with the 26.7-ppm shift observed on reduction of the free flavin (in 100% methanol). The additional 10.9 ppm shift is more than one-third that seen upon formal two-electron reduction. This finding suggests that the benzene end of the flavin is primed to mediate electron transfer from reduced flavodoxin, consistent with earlier proposals (Ludwig et al., 1975; Hemmerich, 1977). Similar conclusions can be drawn from the ^{13}C NMR chemical shifts at flavin positions 6–9, reported with all species of reduced flavodoxins studied (Vervoort et al., 1986).

For *p*-hydroxybenzoate hydroxylase, high-resolution X-ray crystal studies on both the oxidized and reduced forms have been reported for the substrate (*p*-hydroxybenzoate) complexes of the enzyme but not for the uncomplexed enzyme (Wierenga et al., 1979; Schreuder et al., 1987; Schreuder, 1988). From Table I, it can be seen that a larger upfield shift occurs upon reduction of the 8-fluoro-FAD *p*-hydroxybenzoate hydroxylase–*p*-hydroxybenzoate complex than for the uncomplexed enzyme, 8.7 ppm compared to 6.3 ppm. On the basis of the large shift, we would anticipate substantial rearrangement of the flavin–protein interactions upon reduction. However, the X-ray results showed no significant change in the flavin, substrate, or protein positions (Schreuder et al., 1987; Schreuder, 1988). Kinetic studies have shown a large decrease in both the rate of substrate binding and dissociation in the reduced enzyme compared to the oxidized enzyme without any significant change in the K_d (Entsch et al., 1976; Husain & Massey, 1979). These kinetic results can only be interpreted in terms of a conformational change on reduction of the flavin which hinders access of substrate to the active site. Such a structural change would be consistent with our ^{19}F NMR findings. Furthermore, ^{15}N NMR investigations revealed a substantial change in the accessibility of the flavin upon reduction (Vervoort, 1986).

Ligand Binding Perturbations on the 8-Fluoroflavin Chemical Shift

Anthranilate Hydroxylase. Oxidized anthranilate hydroxylase binds anthranilate to form a stable binary complex, K_d = 30 μM (0.05 M potassium phosphate buffer, pH 7.4, 3 °C) (Powlowski et al., 1989). The normally resolved features of the absorbance spectrum are smoothed in the complex [see Figure 2 in Powlowski et al. (1987)]. 8-Fluoro-FAD anthranilate hydroxylase also forms a complex with anthranilate (K_d = 60 μM; 0.1 M potassium phosphate buffer, pH 7.0, 25 °C) with changes in the absorbance spectrum very similar to those seen with native enzyme (data not shown). The ^{19}F

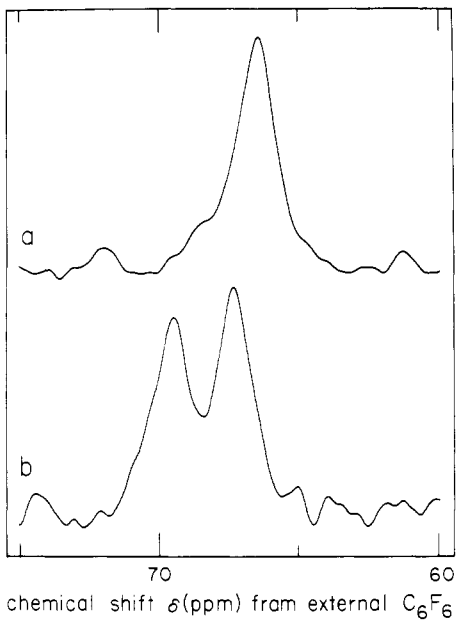


FIGURE 3: Comparison of the ^{19}F NMR spectra for oxidized 8-fluoro-FAD anthranilate hydroxylase, plus and minus anthranilate. Conditions: 190 μM 8-fluoro-FAD anthranilate hydroxylase in 0.1 M potassium phosphate buffer, pH 7.0, 25 °C, was measured in (a) the absence and (b) the presence of 900 μM anthranilate (recrystallized three times from water). The spectra were processed with an exponential line broadening of 300 Hz.

NMR spectrum of this complex (Figure 3) shows two peaks (69.6 and 67.4 ppm), both shifted downfield from that of the uncomplexed enzyme (66.5 ppm). These peaks were observed in the same relative proportions in the presence of either 0.9 or 12 mM anthranilate, arguing against an effect due to excess substrate binding, a phenomenon common to flavoprotein hydroxylases. Recrystallization of anthraniliate (three times from water) did not change the results, indicating that the second peak was not due to a contaminant in the ligand. Thus, the appearance of two peaks in the ^{19}F NMR spectrum most likely indicates the presence of two enzyme–substrate populations. From the area of the peaks, the populations exist in a ratio of ~60:40. Previous kinetic studies (Powlowski et al., 1989) implicated the presence of two oxidized enzyme–substrate complexes, in a ratio of ~70:30 (calculated from the reported rates for equilibrium of these species). We believe that our NMR results provide a direct, physical confirmation of these earlier kinetic interpretations.

In an experiment on reduced enzyme, the anthranilate complex gave only a single peak (Table I). The oxidized enzyme in complex with the substrate analogue salicylate (0.5 or 3.0 mM) also gave only a single peak, shifted slightly downfield to 66.9 ppm.

D-Amino Acid Oxidase. Oxidized D-amino acid oxidase binds benzoate (K_d = 1.3 μM in 0.1 M sodium pyrophosphate/acetate, pH 8.5, 19 °C), which causes its absorbance spectrum to become highly resolved (Quay & Massey, 1977). A similar behavior was found for benzoate binding to 8-fluoro-FAD D-amino acid oxidase ($K_d \leq 5$ μM in 20 mM sodium pyrophosphate, pH 8.5, 25 °C). Benzoate binding caused the ^{19}F NMR peak to be shifted downfield (to 62.5 ppm) and the line width to be remarkably decreased (see Table I). The decrease in line width suggests a ligand-induced depolymerization of D-amino acid oxidase, consistent with earlier reports (Antonini et al., 1966).

General Acyl-CoA Dehydrogenase. The oxidized enzyme binds a variety of substrate analogues, forming long-wavelength-absorbing charge-transfer complexes. We mixed the analogue acetoacetyl-CoA (8 mM) with 8-fluoro-FAD general acyl-CoA dehydrogenase to form a charge-transfer complex with maximal long-wavelength absorbance at 548 nm. The net effect on the ^{19}F NMR signal was a 1.6-ppm upfield shift. Transfer of electron density from acetoacetyl-CoA to the protein-bound 8-fluoro-FAD would be expected to cause an upfield shift.

Old Yellow Enzyme. This enzyme is noted for forming charge-transfer complexes with phenolic ligands (Abramowitz & Massey, 1976a; Massey et al., 1984). We mixed 8-fluoro-FMN Old Yellow Enzyme with a series of para-substituted phenols spanning the range of Hammett σ para-substituent constants (Ritchie & Sager, 1964): (a) *p*-nitrophenol, (b) *p*-hydroxybenzoate, (c) *p*-hydroxybenzaldehyde, (d) *p*-chlorophenol, (e) *p*-methylphenol, and (f) *p*-methoxyphenol. In each case, saturating concentrations of ligand were used and a long-wavelength-absorbance band was recorded (see Table I). The ^{19}F NMR peaks for the complexes showed increasingly larger upfield shifts as the electron-donating capacity of the phenolic ligand increased (Figure 4 and Table I). A plot of the NMR peak positions versus the Hammett σ para-substituent values (Figure 5) illustrates this correlation. The upfield shift is in the direction expected for increasing electron density in the isoalloxazine ring. A similar correlation between ^{15}N chemical shifts and the pK_a of the phenol has been reported by Beinert et al. (1985b) from experiments on Old Yellow Enzyme–phenolate complexes.

Plotting the long-wavelength-absorbance maxima of the charge-transfer complexes versus the Hammett σ para-substituent constants for each ligand also gave a linear correlation (data not shown). Similar linear correlations have been described for native Old Yellow Enzyme and for enzyme substituted with 7-Br-FMN and 5-deaza-FMN Abramowitz & Massey, 1976b).

Two of the ligands used in this study (*p*-chlorophenol and *p*-hydroxybenzaldehyde) were used earlier to investigate the effect of changing the electron-accepting capacity of the flavin on the absorbance properties of the charge-transfer complex (Abramowitz & Massey, 1976b). In that work, apo-Old Yellow Enzyme was reconstituted with a series of modified flavins of varying redox potential. The energy of the long-wavelength transition (expressed as wavenumber) systematically decreased as the electron-accepting capacity of the flavin (expressed as redox potential) became weaker [see Figure 3 in Abramowitz and Massey (1976b)]. We found that 8-fluoro-FMN Old Yellow Enzyme data fit accurately onto that plot with either *p*-chlorophenol or *p*-hydroxybenzaldehyde as the ligand (data not shown). For this comparison, we used a redox potential of −180 mV for 8-fluororiboflavin, which was determined by Dr. R. Stewart (in this laboratory), using standard electrochemical procedures (Stewart & Massey, 1985). This value is significantly different from the previously reported value of 0 mV (McCormick et al., 1963) but similar to the potential of the closely related 8-chloro-FMN, −150 mV (Stewart & Massey, 1985).

The NMR spectrum for many of the complexes showed two clearly distinguishable peaks (see Figure 4). Separation of the peaks was less distinct in some spectra, though shoulders in these cases still suggested the existence of multiple, overlapping peaks. Purification of the phenolic ligands (*p*-hydroxybenzoate by recrystallization from water and *p*-methoxyphenol by sublimation) did not prevent the appearance of multiple peaks, arguing against artifacts due to contaminants in the ligand. The multiple peaks appear to represent multiple flavin-binding environments induced by ligand

Table II: ^{19}F NMR Parameters for Fluorinated Phenols Bound to Old Yellow Enzyme[a]

OYE form	ligand	concn OYE (μM)	concn phenol (μM)	peak position (ppm) [line width (Hz)][b]				no. of scans
	p-F-phenol		sat.[c]	38.0 [2.3]				64
native	p-F-phenol	390		38.1 [251]	30.1 [184]			28912
	2,4-diF-phenol		sat.[c]	40.9 [5]	29.8 [5]			64
native	2,4-diF-phenol	390	390[d]	31.9 [122]	30.9 [196]	30.3 [127]	27.9 [113]	12640
isozyme 3	2,4-diF-phenol	135	135[d]	31.9	30.9	30.3 [236]	27.9 [110]	19168
isozyme 5	2,4-diF-phenol	130	130[d]	31.9 [92]	30.9 [153]	30.4	27.8	31260

[a] All measurements were made in 0.1 M KP_i buffer, pH 7.0, 25 °C. [b] Underlined peaks were present as minor components; see Figure 6. [c] Measurements were made on a saturated solution of fluorophenol in 0.1 M potassium phosphate buffer, pH 7.0, 25 °C. [d] Binding of 2,4-difluorophenol was stoichiometric under the conditions for these samples (K_d about 0.5 μM).

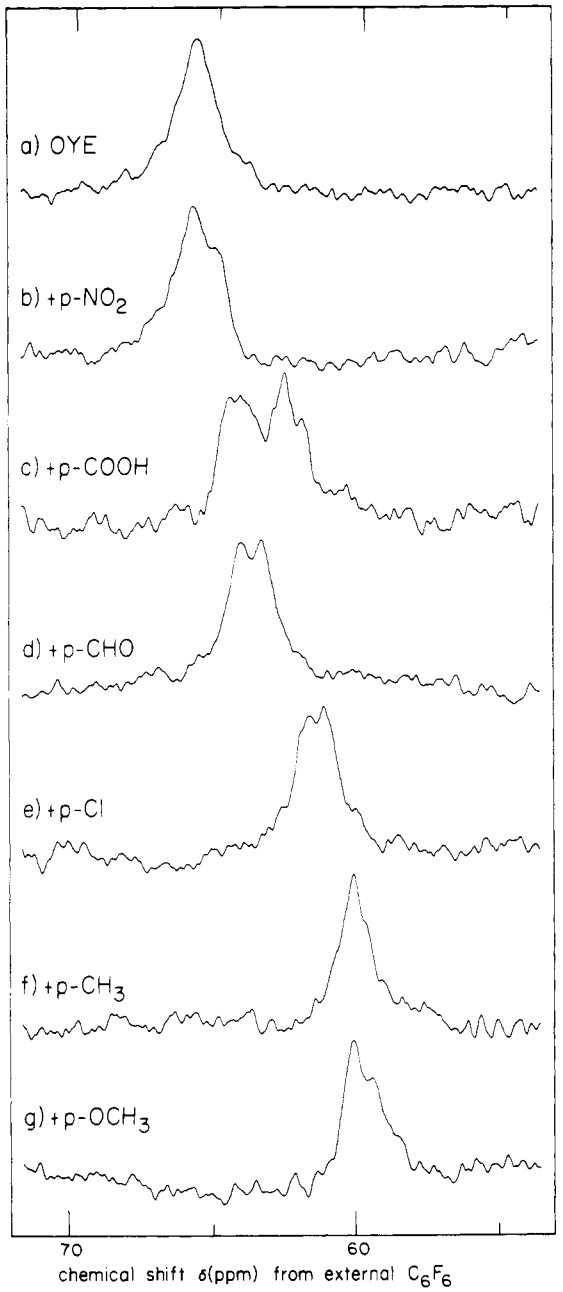


FIGURE 4: ^{19}F NMR spectra of oxidized 8-fluoro-FMN Old Yellow Enzyme plus and minus phenolic ligands. Conditions: 250–300 μM 8-fluoro-FMN Old Yellow Enzyme in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C, was mixed with saturating concentrations of para-substituted phenols as indicated: (a) OYE, no ligand; (b) +p-NO_2, *p*-nitrophenol; (c) +p-COOH, *p*-hydroxybenzoate; (d) +p-CHO, *p*-hydroxybenzaldehyde; (e) +p-Cl, *p*-chlorophenol; (f) +p-CH_3, *p*-cresol; (g) +p-OCH_3, *p*-methoxyphenol. Spectra are displayed from top to bottom in order of increasing electron-donating capacity of the phenol. The spectra were processed with an exponential line broadening of 100 Hz.

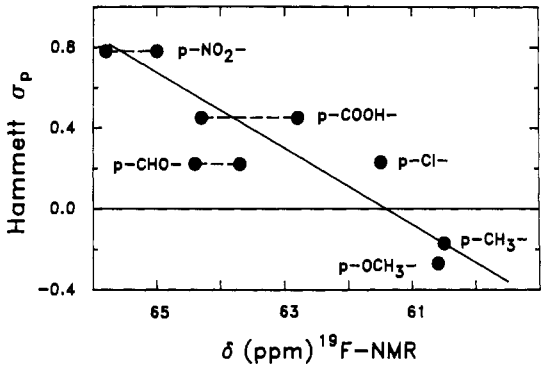


FIGURE 5: Plot of the ^{19}F NMR peak positions for the 8-fluoro-FMN Old Yellow Enzyme–phenolate complexes versus the Hammett σ para-substituent constant for each phenol. Conditions: same as in Figure 4. Two points are plotted for those complexes showing two distinct peaks in the NMR spectrum (see Figure 4).

binding. Multiple environments can be attributed to the multiple isozymes known to be present in Old Yellow Enzyme, as isolated by affinity chromatography (Miura et al., 1986).

Fluorinated Ligands Bound to Native Old Yellow Enzyme

p-Fluorophenol, free in solution, showed a single ^{19}F NMR peak at 38.0 ppm (Table II). This ligand binds to Old Yellow Enzyme (K_d = 125 μM; 0.1 M potassium phosphate buffer, pH 7.0, 25 °C) to form a charge-transfer complex with a long-wavelength maximum at 645 nm. The complex gave two peaks (38.1 and 30.1 ppm) in the ^{19}F NMR spectrum. Both peaks were broad (see Table II), suggesting that they both represented protein-bound ligands, thus indicating two different ligand-binding environments. However, the facts that the downfield peak was located at the same position as the free ligand and that the binding of *p*-fluorophenol to Old Yellow Enzyme was relatively weak made us hesitant to interpret the 38.1-ppm peak as a protein-bound resonance. We therefore turned to 2,4-difluorophenol.

2,4-Difluorophenol free in solution gave two ^{19}F NMR peaks (40.9 and 29.8 ppm). Comparing these positions to that of *p*-fluorophenol, we can assign the 40.9-ppm peak to the fluorine at position 4, leaving the 29.8-ppm peak for position 2. This ligand binds tightly to Old Yellow Enzyme (K_d about 0.5 μM; 0.1 M potassium phosphate buffer, pH 7.0, 25 °C) giving a charge-transfer complex with a long-wavelength maximum at 640 nm (ϵ = 4500 M^{-1} cm^{-1}). The complex gave four peaks in the ^{19}F NMR spectrum (31.9, 30.9, 30.3, and 27.9 ppm) (see Figure 6 and Table II), none of which overlapped with the peak positions of the free ligand. A shoulder at 29.8 ppm and a small peak at 40.9 ppm suggested the existence of a small portion of free ligand. The two new pairs of peaks clearly indicate the existence of two separate ligand-binding environments.

Beinert et al. (1985a) demonstrated the presence of multiple isozymes in Old Yellow Enzyme by isoelectric focusing, and

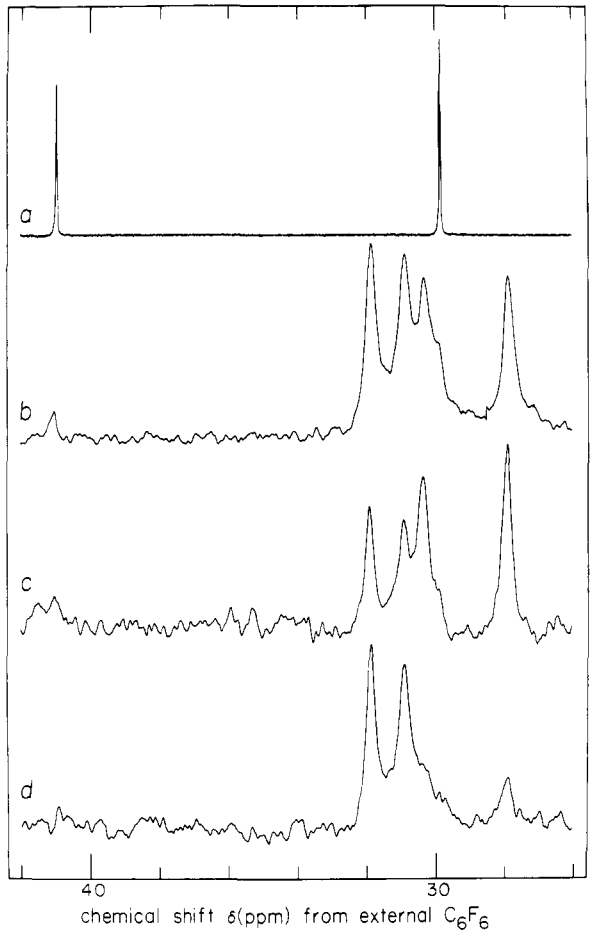


FIGURE 6: ^{19}F NMR spectra of 2,4-difluorophenol, free and bound to native Old Yellow Enzyme. Conditions: 0.1 M potassium phosphate buffer, 25 °C. Spectra are of 2,4-difluorophenol: (a) free in solution; (b) in complex with affinity-purified Old Yellow Enzyme isozyme mixture (390 μM); (c) in complex with FPLC-purified Old Yellow Enzyme isozyme 3 (135 μM); (d) in complex with FPLC-purified Old Yellow Enzyme isozyme 5 (130 μM). Under the conditions of these experiments, 2,4-difluorophenol is bound stoichiometrically to Old Yellow Enzyme and is therefore present in approximately equal concentration to the enzyme in each case. Spectra were processed with an exponential line broadening of 50 Hz, except for (a) which was processed with 1.5 Hz.

these were independently observed and separated by FPLC chromatography on Mono-Q media (Miura et al., 1986). Miura et al. (1986) using ^{13}C NMR presented evidence for two flavin-binding environments in Old Yellow Enzyme and were able to demonstrate that different isozymes were responsible for the different flavin-binding environments. Following the procedure of Miura et al., we were able to resolve affinity-purified Old Yellow Enzyme into five isozymes, three of which were major components but only two of which were isolated in amounts sufficient for NMR measurements. Using their numbering scheme, isozyme 5, when complexed to 2,4-difluorophenol, gave only two major peaks in the ^{19}F NMR spectrum (31.9 and 30.9 ppm) (Figure 6). The ^{19}F NMR spectrum of the complex with isozyme 3 showed the presence of all four resonances of the initial enzyme mixture but was dominated by the pair of peaks at 30.3 and 27.9 ppm. Thus, the NMR results suggest that isozyme 3 is associated with peaks at 30.3 and 27.9 ppm, while isozyme 5 is associated with peaks at 31.9 and 30.9 ppm, leading to the conclusion that the ligand-binding environment is different for each of these two isozymes. Earlier ^{13}C NMR work found that the flavin-binding environment of isozyme 1 was different from that of isozyme 5 (Miura et al., 1986). These isozyme-dependent differences in Old Yellow Enzyme suggest that the ligand-induced splitting of the 8-fluoro-FMN Old Yellow Enzyme ^{19}F NMR peaks, described in a previous section (cf. Table I), can also be attributed to differences in the isozyme subpopulations of the enzyme.

In the ^{19}F NMR spectrum of the isozyme 5 complex, the area of the small peaks at 30.4 and 27.8 ppm represented about 10% of the total signal. This is significantly greater than the approximately 1% contamination indicated by the FPLC elution profile for this isozyme as originally isolated. Furthermore, the minor peaks, at 31.9 and 30.9 ppm, in the ^{19}F NMR spectrum of the isozyme 3 complex were 28% of the total. This was much greater than expected for that isozyme, which also showed about 1% contamination as originally isolated. We therefore rechromatographed both isozymes on Mono-Q after the NMR measurements. Isozyme 5 was found now to be about 13% enriched in isozyme 3, consistent with the NMR measurement. Isozyme 3 contained about 21% of isozyme 1 and 18% of isozyme 5. Thus, it appears that the purified isozymes partially reconvert to mixtures during handling. Furthermore, it appears as though isozyme 5 can convert directly to isozyme 3 but it cannot convert directly to isozyme 1, whereas isozyme 3 can convert directly into either isozyme 1 or isozyme 5. Because of the interconvertibility, it is clear that there are no differences in the primary structure of the isozymes. It seems reasonable to ascribe the different isozymes to multiple metastable conformations of the native protein which can be detected both by ion exchange chromatography and by NMR.

Conclusions

We have found ^{19}F NMR to be a sensitive technique for studying flavoproteins. Four-tenths of a milliliter of sample containing 140–450 μM protein-bound flavin was sufficient to obtain good signals (Figure 1) from proteins as large as 350 kDa (L-lactate oxidase).

The oxidized 8-fluoro flavoprotein peaks were clustered around the position of 8-fluororiboflavin (64.9 ppm, relative to hexafluorobenzene), being found upfield as much as 5.6 ppm (8-fluoro-FAD D-amino acid oxidase) and downfield as much as 4.5 ppm (8-fluoro-FAD glucose oxidase), see Table I. Reduction of the flavin caused a major upfield shift (26.7 ppm for 8-fluororiboflavin in 100% methanol), leaving the peaks of the protein-bound flavin spread over a similar range (10.3 ppm). In addition to the major upfield shifts induced by reduction, secondary shifts were observed with several of the 8-fluoro flavoproteins (Figure 2). The largest of these secondary shifts occurred with 8-fluoro-FMN flavodoxin, which was shifted an additional 10.9 ppm upfield. This additional shift denotes significantly greater electron density in the benzene ring of the reduced protein-bound flavin, indicating that this portion of the isoalloxazine is primed to mediate electron transfer from reduced flavodoxin as has been suggested previously (Ludwig et al., 1975; Hemmerich, 1977).

Ligand binding typically caused a shift in the NMR peak position for both the oxidized and reduced 8-fluoro flavoproteins. With oxidized 8-fluoro-FMN Old Yellow Enzyme, binding of phenolates resulted in progressively greater upfield shifts as the electron-donating capacity of the phenolate increased (Figure 5). These ligands serve as charge-transfer donors to the bound oxidized FMN of Old Yellow Enzyme (Massey et al., 1984). The upfield direction of the shifts of the NMR resonance indicates that the increasing electron density in the isoalloxazine ring system is the overriding factor in defining the observed chemical shift changes. Thus we have a clear example of charge-transfer interactions perturbing the

^{19}F NMR spectrum of a flavin. An analogous upfield shift was found for the 8-fluoro-FAD general acyl-CoA dehydrogenase–acetoacetyl-CoA charge-transfer complex (Table I).

^{19}F NMR has also proved useful in delineating ligand-binding phenomena. A split in the oxidized 8-fluoro-FAD anthranilate hydroxylase peak was induced upon binding of anthranilate (Figure 3), to give two populations (60:40) which corresponded well with the kinetic model for anthranilate hydroxylase activity (Powlowski et al., 1989). Finally, we were able to utilize fluorinated ligands to examine the ligand-binding environments of the protein. 2,4-Difluorophenol clearly illustrated an isozyme-dependent heterogeneity in the ligand-binding domain (Figure 6) of native Old Yellow Enzyme.

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Structure of Interleukin 1α at 2.7-Å Resolution†

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ABSTRACT: The interleukin 1 (IL-1) family of proteins has a central role in modulating immune and inflammatory responses. Two major IL-1 proteins, designated α (IL-1α) and β (IL-1β), are produced by activated macrophages and other cell types. In an effort to understand the similarities and differences in the physicochemical and functional properties of these two proteins, a program was initiated to determine their structures. Crystals of IL-1α were grown, and the three-dimensional structure at 2.7-Å resolution was solved. The technique of multiple-wavelength anomalous dispersion (MAD) with the selenomethionine form of IL-1α was utilized in combination with a single mercury derivative to provide the starting phases. Partial refinement of the IL-1α model has been performed as well. The overall structure is composed of 14 β-strands and a 3_{10} helix. The core of this structure is a capped β-barrell that possesses 3-fold symmetry and displays a topology similar to that observed for IL-1β [Priestle, J. P., et al. (1988) *EMBO J. 7*, 339–343] and soybean trypsin inhibitor (STI) [McLachlan, A. D. (1979) *J. Mol. Biol. 133*, 557–563]. In this paper, the overall structure of IL-1α and the nature and fidelity of the internal 3-fold symmetry are discussed. Comparisons with IL-1β and STI are made within these contexts.

Interleukin 1 (IL-1) molecules found in serum are relatively small proteins of approximately 17 500 daltons and are thought to be involved in the initiation or exacerbation of a number of disease states including rheumatoid arthritis (Dinarello, 1984; Oppenheim et al., 1986; Lomedico et al., 1986). The cDNAs encoding human IL-1α and IL-1β have been sequenced (Auron et al., 1984; March et al., 1985; Gubler et al., 1986), showing that both proteins are synthesized as larger precursor molecules. The primary structures of the IL-1α and IL-1β precursors have several common features: (1) they are approximately the same size (271 and 269 amino acids, respectively), (2) they lack a classical signal peptide sequence, and (3) the IL-1 bioactivity maps to the carboxy-terminal half of the precursor. Among various mammalian species, IL-1α proteins are all between 60% and 70% identical in sequence to one another. Similar sequence identity has been noted for the IL-1β proteins. However, there is less than 30% sequence identity between IL-1α and IL-1β proteins. Despite marked differences in amino acid sequences, both IL-1α and IL-1β appear to have similar, if not identical, biological activities (Rupp et al., 1986) as they bind to the same receptor protein (Dower et al., 1986a,b; Kilian et al., 1986; Matsushima et al., 1986) with similar affinities on target cells. IL-1α and IL-1β do differ in certain physicochemical properties [e.g., isoelectric points (Schmidt, 1984; Saklatvala et al., 1985) and sensitivity to heat (Krakauer, 1985)], but they exhibit similar NMR and CD spectra (Gronenborn et al., 1986; Craig et al., 1987; D. C. Fry, personal communication).

We have undertaken the solution of the three-dimensional structure of a recombinant form of human IL-1α as part of a drug discovery program. We have also used this project in an effort to demonstrate the feasibility of utilizing selenomethionine forms of proteins with the multiple-wavelength anomalous dispersion (MAD) method for solving protein structures without heavy-atom derivatives (Hendrickson, 1985). We report herein the crystal structure of IL-1α at 2.7-Å resolution.

Experimental Procedures

The purification of IL-1α followed standard chromatographic techniques as detailed elsewhere (Roy et al., 1989).

† The Cα coordinates have been deposited in the Brookhaven Protein Data Bank (code 1ILA).

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