**19F NMR Studies with 2'-F-2'-deoxyarabinoflavoproteins**

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Apoproteins of several flavoproteins were reconstructed with 2'-F-2'-deoxyarabinoflavins and studied by 19F NMR and absorption spectroscopy. Extensive protein-fluorine interactions were observed by large chemical shift changes on binding to the apoprotein of Old Yellow Enzyme (apoOYE) and apoflavodoxin, whereas binding to apoglucose oxidase and apo d-amino acid oxidase (apoDAAO) resulted in minimal interactions. Modification at the flavin 2'-position in OYE resulted in a substantial decrease in the binding affinity of the flavin, possibly from the disruption of two important hydrogen bonds to Pro-35 and Arg-243. 19F NMR studies of complexes of OYE with testosterone, cyclohexenone, and β-estradiol suggest that phenols and α,β-unsaturated ketones orient differently at the active site on binding. The two separate one-electron potentials for the EFsq/EFred and EFsq/EFred couples were different for the reconstructed OYE. With native enzyme, there is 15-20% thermodynamic stabilization of the anionic flavin semiquinone, while no detectable amount of semiquinone was observed with modified OYE. This change in potential was further substantiated by blue shifts for the maxima of the modified protein-phenol charge transfer complexes. In accordance with the crystal structure of the OYE-p-OH-benzaldehyde complex (Fox, K.M. & Karplus, P.A. (1994) Structure 2, 1089–1105), 19F NMR studies with the modified OYE-2,4-F2-phenol suggest strong interaction between the para-fluorine of the phenol and Tyr-375.

In order to understand the various mechanisms of flavin reactions, knowledge of the protein environment at the active centers of different flavoenzymes is necessary. Several physical and chemical methods are available for this purpose. Of these, replacement of the native flavin with appropriately modified flavins and high resolution NMR techniques constitute very effective and widely exploited tools. The former method in general provides information about the solvent accessibility of the modified position, hydrophobicity, and steric constraints of the binding site (1). In the latter technique, comparison of the chemical shifts of the protein-bound flavin with the free flavin as well as the oxidized and reduced flavoproteins provides direct information about alterations in the magnetic environment of the substituent fluorine and hence can provide information about the protein environment surrounding the flavin (2-4). Old Yellow Enzyme is the oldest of the flavoprotein family and, as isolated from brewer's bottom yeast, is a mixture of homodimers and a heterodimer arising from two separate yeast genes, with each monomeric unit containing one FMN (5, 6). Due to the fact that the physiological role of this enzyme is yet to be determined, the structure and reactivity of this protein has been the subject of extensive studies in our laboratory. Recent x-ray crystallographic studies suggest an important role for the 2'-hydroxyl group of the FMN in binding to the apoprotein and also in forming the binding site for various ligands (7, 8). This hydroxyl group is involved in two important hydrogen bonds with Pro-35 and with Arg-243, one of the positively charged residues around the flavin N1 (Fig. 1). It was thought that the disruption of these hydrogen bonds would affect the configuration of the net charge at the binding site and so it was of obvious interest to study how this would affect the flavin binding to apoprotein, binding of various ligands, and catalytic activity. A new flavin, 2'-fluoro-2'-deoxyarabinobilin was designed, where the 2'-hydroxyl group of the ribityl side chain was replaced with a hydrogen and a fluorine was incorporated in place of the hydrogen at the same 2'-carbon (9, 10). This flavin should be an ideal probe for ligand-binding studies and also provide information on the chemical environment of the flavin binding site in terms of 19F chemical shifts. It was also thought that this flavin could be a good 19F NMR probe for flavoproteins in general. The high sensitivity of 19F, which is next only to 1H, the wide range of chemical shifts, and the ease with which one can incorporate fluorine into biological molecules make 19F NMR a very effective physical tool to study proteins (11-13). Another important feature is that the number of peaks for each fluorine correspond to the number of binding environments present in the protein. Since the fluorine is in the ribityl side chain of this flavin, the net electron environment (fluorine is highly electronegative) of the isoalloxazine ring of the flavin is unperturbed by its presence, unlike in the case of 8-F-flavins, where the benzene ring of the flavin experiences more positive character due to the presence of the electronegative fluorine (4). Another advantage of fluorine being on the hydrophilic ribityl side chain is that it interacts more with the protein; and changes in the fluorine chemical shifts are expected to be very sensitive to capture even minute changes in the protein conformation. In the case of 8-F-flavin, fluorine being on the hydrophobic benzene ring of the flavin, the differences in chemical shifts are not that pronounced for most of the proteins studied (4).

**EXPERIMENTAL PROCEDURES**

**Materials—**d(+)-Glucose was from ICN. Deuterium oxide and 2-fluoro-2-deoxy-1,3,5-tri-O-benzoyl-α-D-arabinofuranose were from Sigma, p-Fluorophenol, o-fluorophenol, 2,4-difluorophenol, p-chlorophenol, p-hydroxybenzaldehyde, 4-amino-o-xylene, barbituric acid were from Aldrich.

Preparation of Flavins—2'-F-arabinoflavin and 2'-deoxyriboflavin were synthesized and converted to the FAD and FMN levels as reported earlier (9).

Preparation of Flavoproteins and Their Corresponding Apoproteins—

The holoproteins and apoproteins were prepared as described prevously: flavodoxin from Megasperma elsdenii (14, 15), Old Yellow Enzyme (16, 17), glucose oxidase from Aspergillus niger (18, 19), and d-amino acid oxidase from pig kidneys (20).

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Changes over a period of a few months when stored at highly stable over prolonged periods, showing no detectable identity of free as well as protein-bound flavins was studied. It was however, when the proteins were reduced with sodium dithionite, all samples were prepared in 50 mM potassium phosphate buffer, pH 7.0.

Flavoproteins with 2'-F-2'-deoxyflavins

Flavodoxin—The binding of 2'-F-arabino-FMN to apo-flavodoxin was followed both by fluorescence and by absorption spectra. At the end of the titration the flavin fluorescence was almost fully quenched with a residual fluorescence ~2.5% and with the \( \lambda_{\text{max}} \) changed from 372 and 444 nm for free flavin to 378 and 446 nm for the protein-bound flavin. The extinction coefficient for 2'-fluoro FMN was determined as 11,300 M\(^{-1}\) cm\(^{-1}\) by standardizing the apoprotein with pure normal FMN. From the titration plots, the \( K_d \) was determined as 1.2 ± 0.2 \( \times 10^6 \) M (4–5 \( \times 10^{-6} \) M for native flavin), suggesting that the absence of the 2'-hydroxyl group weakened considerably the binding of the flavin to the protein. The \( ^{19} \)F NMR spectrum of the 2'-fluoro-FMN-reconstituted apoflavodoxin showed a single peak at 74.6 ppm, about a 10-ppm downfield shift from the free flavin resonance, suggesting extensive flavin protein interactions in this region of fluorine. In the clostridial flavodoxin, the 2'-OH group lies 2.6 Å away from Ala-55 and forms a strong hydrogen bond with the carbonyl of the alanine (24, 25). The sodium dithionite-reduced modified protein has a peak at 64.55 ppm, almost the same as the free flavin chemical shift. This suggests that in the reduced form of the flavoprotein, fluorine interactions with the protein are minimal (Table I).

Old Yellow Enzyme—The binding of apoOYE with 2'-F-arabinoflavin was followed by measuring the flavin fluorescence. At the end point, the fluorescence was almost completely quenched, with a residual intensity ~3% that of the original. From the titration plots, the \( K_d \) was determined as 4.6 ± 0.3 \( \times 10^{-6} \) M, which is 10^4 times weaker binding than the native flavin (\( 10^{-2} \) M). The \( \lambda_{\text{max}} \), for free flavin to protein-bound flavin shifted from 372 and 444 nm to 378 and 452 nm. The substantial decrease in binding affinity is quite understandable, since the flavin modification disrupts two hydrogen bonds that the 2'-hydroxyl group makes with Pro-35 and Arg-243 (Fig. 1).

The \( ^{19} \)F NMR spectrum of the oxidized enzyme has a peak at 80.75 ppm, a downfield shift of 15 ppm from the free flavin resonance (Fig. 2). This kind of large downfield shift generally means an increased electron density near the fluorine or a strong hydrogen bond formation involving fluorine. The \( ^{19} \)F resonance for the reduced enzyme, reduced with sodium dithionite, was recorded at 64.65 ppm, a large upfield shift of 16 ppm from the oxidized form toward the free flavin region (Fig. 2). This means that the strong fluorine-protein interactions in the oxidized form, which are indicated by the large downfield shift, are lost on reduction and that the fluorine signal is shifted upfield almost to the free flavin region. Interestingly, the crystal structure does not reveal significant changes in the flavin-protein hydrogen bonding interactions on reduction (8). However, reduction was accompanied by two interesting changes in the flavin conformation. In the reduced state, the flavin attains a butterfly conformation associated with puckering at N-5 and the FMN ribityl chain shifts from one staggered conformation to the other (8). As a consequence, the interactions between the fluorine and the protein in the reduced form might have been disrupted, resulting in the observed upfield shift to the free flavin region (Table I).

**RESULTS AND DISCUSSION**

Stability of Modified Flavins and Flavoproteins—The stability of free as well as protein-bound flavins was studied. It was found that both the 2'-F-flavins and the 2'-deoxyflavins are highly stable over prolonged periods, showing no detectable changes over a period of a few months when stored at ~20 °C. However, when the proteins were reduced with sodium dithionite, a small amount of free fluor ide was seen in the \( ^{19} \)F NMR spectra.

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1 The abbreviations used are: OYE, Old Yellow Enzyme; DAAO, \( \alpha \)-amino acid oxidase.
 modification of the flavin on the binding of these ligands was studied by means of the changes in spectral properties and $^{19}$F NMR measurements.

The native enzyme binds to p-chlorophenol with a $K_d$ of $-1 \mu M$ and results in a new peak with a maximum at 645 nm. Many lines of evidence indicate that this long wavelength absorbance represents charge transfer interaction wherein the phenolate is the donor and the flavin the acceptor (8, 16, 17). When the 2'-phenolate is the donor and the flavin the acceptor (8, 16, 17, 18), sorbate represents charge transfer interaction wherein the configuration of the charge around the flavin, since an important hydrogen bond, which holds Arg-243 with the 2'-hydroxyl group of the native FMN is now nonexistent. To expand this observation, the binding of various structurally diverse phenols was studied.

2,4-Difluorophenol binds tightly to native enzyme with a $K_d$ of about 0.5 $\mu M$ and results in a long wavelength band with a maximum at 640 nm. The $^{19}$F NMR spectra of this complex with native enzyme showed two sets of signals for the ligand and were assigned for the two isozymes of the enzyme (4). We decided to study this ligand with the modified flavoprotein, since the $^{19}$F resonances of the complex from the ligand and the flavin might reiterate this fact. The titration plots suggested binding with a $K_d$ of 2 $\mu M$, and the maximum of the long wavelength band was observed at 604 nm, shifted by 36 nm toward lower wavelength (Fig. 3). This is consistent with the result we obtained with the modified flavoprotein.

The $^{19}$F NMR spectrum of the free 2,4-difluorophenol has two resonances for the two fluorine atoms at 44.15 and 31.85 ppm. From the spectra of $p$- and $o$-fluorophenols, the peak at 44.15 ppm was assigned to the fluorine present at the para-position and the other at 31.85 ppm to the fluorine at the ortho-position. The $^{19}$F spectrum of the ligand bound to the protein showed seven resonances (Fig. 4; Table I). Of these the two at 44.17 and 32.1 ppm appear to be due to the unbound ligand. The peak heights of these two signals, in comparison with the resonances from the bound ligand, suggest fast association and dissociation of the ligand with the protein. The bound ligand has two sets of peaks at 34.35, 32.95, 33.45, and 30.95 ppm, which is in accordance with the known fact that the enzyme from brewers’ yeast is a mixture of two isozymes (6). Interestingly, the fluorine from the flavin shows only one peak at 79.86 ppm, about a 1-ppm shift from that of the ligand-free protein. This indicates that the binding of 2,4-difluorophenol does not result in any major rearrangement in the protein conformation. From the resonances of the bound ligand, it can be seen that the peak from the fluorine that is in the $o$-position with the hydrogen of the $-OH$ group of Tyr-375, which was recently determined by Fox and Karplus (8).

To further substantiate the interaction of the fluorine in the para-position with Tyr-375, we have recorded the $^{19}$F NMR spectra for the complexes of the modified protein with $p$- and $o$-fluorophenols. It was found that the 2'-F-FMN-OYE binds with $o$-F-phenol at pH 7, and the maximum of the long range band was observed at 554 nm. This is a shift toward lower wavelength region by 56 nm from the native protein band with a maximum at 610 nm, shifted 35 nm down from that with native enzyme (Table I). This shows that the present modification has affected both the binding of the phenol and the resulting spectral properties. This could reasonably be ascribed to a change in the configuration of the charge around the flavin, since an important hydrogen bond, which holds Arg-243 with the 2'-hydroxyl group of the native FMN is now nonexistent. To expand this observation, the binding of various structurally diverse phenols was studied.

Flavoproteins with 2'-F-2'-deoxyflavins

| Oxidized | Reduced |
|----------|---------|
| ppm      | ppm     |
| 2'-F-FAD | 65.4    | 66.3    |
| 2'-F-FMN | 65.75   | 66.3    |
| 2'-F-FMN-flavodoxin | 74.6 | 64.55 |
| 2'-F-FAD-glucose oxidase | 66.38 | 70.40 |
| 2'-F-FAD-o-aminoo acid oxidase | 65.65 | 64.95 |
| 2'-F-FAD + benzene | 65.35 |
| 2'-F-FAD + sulfate | 65.45 |
| 2'-F-FMN-OYE | 80.75 | 64.65 |

TABLE I
$^{19}$F NMR chemical shifts (ppm) for 2'-F-flavins and 2'-F-flavoproteins

Spectra were recorded in 50 mM KPi, pH 7, and hexafluorobenzene as an external standard. The samples contained 80–150 $\mu M$ enzyme in 350 $\mu L$ of 0.1 M KPi, pH 7, and 50 $\mu L$ of D$_2$O. For making the ligand-bound protein samples, protein was titrated at total volume of 1 ml and then concentrated to 350 $\mu L$ by ultrafiltration with a Centricon 30 microconcentrator.

| Complexes of 2'-F-FMN-OYE with various ligands | Oxidized | Reduced |
|-----------------------------------------------|----------|---------|
| OYE + 2,4-F$_2$phenol | 79.86 (FMN) 44.17, 32.1 | bound phenol) |
| Free 2,4-F$_2$phenol | 44.15, 31.85 |
| OYE + p-ÖH-benzaldehyde | 80.7 |
| OYE + β-estradiol | 80.7 (ligand-free protein), 83.7 (ligand-bound protein) |
| OYE + testosterone | 86.2 |
| OYE + cyclohex-2-en-1-one | 85.3 |
| OYE + p-F-phenol | 41.6 (free ligand), 32.6 (bound ligand), 80.5 (flavin) |
| OYE + o-F-phenol | 31.3 (broad signal), 80.1 (flavin) |
| OYE (FPLC fraction 1) | 80.3 |
| OYE (FPLC fraction 1) + 2,4-F$_2$phenol | 79.75 (flavin), 32.7 and 30.8 (bound ligand) |
| OYE (FPLC fraction 3) | 80.1 |
| OYE (FPLC fraction 3) + 2,4-F$_2$phenol | 79.5 (flavin), 34.3 and 33.4 (bound ligand), 44.1 and 31.85 (free ligand) |

* FPLC, fast protein liquid chromatography.
Flavoproteins with 2'-F-2'-deoxyflavins

from the ligand-free protein. The signal from the protein-bound ligand appeared at 32.6 ppm, a 12-ppm upfield shift from the free ligand, which can be attributed to interaction with Tyr-375 (Table I). In addition to these two signals, a very strong peak appeared for the unbound ligand, suggesting weak binding of p-F-phenol to the modified protein. It has been shown that in brewers’ yeast, p-OH-benzaldehyde is a natural ligand for OYE, and this complex is responsible for the major green form of the protein (28). This ligand binds very tightly to the native protein with a $K_d$ of 0.1 $\mu$M with the maximum of the charge transfer band located at 575 nm. In light of our observations with the above two phenols, it was interesting to study the binding of this phenol to 2'-F-FMN-OYE. Interestingly, the chemical modification at the 2'-position has absolutely no effect on the binding of p-OH-benzaldehyde, apart from the usual shift of the wavelength band to shorter wavelengths (Table II). This was found to bind as tightly to the reconstituted protein as to the native protein, with a $K_d$ of 0.05 $\mu$M (Table II). This can be very well explained by the x-ray structure (Fig. 1), which shows that the aldehyde carbonyl of the ligand hydrogen bonds to the –OH of Tyr-375, providing an additional binding point for this ligand.

The 19F NMR spectrum of the 2'-F-FMN-enzyme has one resonance at 80.7 ppm for the complex of p-OH-benzaldehyde with the reconstituted protein. This is only a 0.5-ppm downfield shift from the ligand-free protein, showing no significant alteration in the magnetic environment of the fluorine and consistent with no major rearrangement in the flavin-protein interactions on binding of this ligand.

It has recently been found that the native enzyme binds very tightly to various steroid molecules (26, 29). We chose two structurally different steroids for our studies with the reconstituted protein. One was testosterone, which has an $\alpha,\beta$-unsaturated moiety. This was selected because of the fact that the native protein reduces various $\alpha,\beta$-unsaturated compounds (6). The other was $\beta$-estradiol, which has a phenolic ring. It was found that testosterone binds tightly to the native protein with a $K_d$ of 1.6 $\mu$M without forming any charge transfer complex. Interestingly, when the 2'-F-FMN-reconstituted protein was titrated with testosterone, it was found to bind tightly also with a $K_d$ of 6 $\mu$M. Apparently, the chemical modification of the flavin has little effect on the binding of this ligand and was accompanied by a large perturbation in the absorption spectrum, as in the case of the native protein. The 19F NMR of this complex has a resonance at 86.2 ppm, a large downfield shift of 5.5 ppm from the ligand-free protein (Fig. 2, Table I).

Native protein also binds tightly to $\beta$-estradiol with a $K_d$ of <1 $\mu$M and forms a charge transfer complex with a maximum located at 670 nm. It was found from the titration that this ligand binds only weakly to the 2'-F-FMN-reconstituted protein with a weak charge transfer transition. The maximum of this long wavelength band was located at 622 nm, a shift toward lower wavelengths of 48 nm, consistent with the results with other phenols.

The 19F NMR spectrum of the complex showed two resonances. The strong peak at 80.7 ppm, from the ligand-free protein, is consistent with a weak binding of $\beta$-estradiol to the modified protein. The ligand-bound protein has a peak at 83.7 ppm, an ~3-ppm downfield shift, suggesting the possibility of some conformational rearrangements in the protein as a result of our observations with the above two phenols.

| Table II | Ligand binding to native OYE and 2'-F-FMN-OYE |

| Wild type | 2'-F-FMN-OYE |
|-----------|-------------|
| Absorbance maximum | 378 and 452 nm |
| $K_d$ | $4.3 \times 10^{-10}$ M |
| Wild type | 2'-F-FMN-OYE |
| CT absorbance maximum | 378 and 452 nm |
| $K_d$ | $4.3 \times 10^{-10}$ M |
| OYE fraction 1* ($K_d$) | 3-4 |
| OYE fraction 3* ($K_d$) | 1.5-2 |

* OYE from brewers’ yeast was resolved into separate isozymes (Ref. 6), and apoprotein was prepared (Ref. 16, 17) and reconstituted with 2'-F-FMN.
of binding of the steroid molecule (Fig. 2, Table I). It was found that cyclohexenone is both an electron donor and acceptor for OYE with phenol and cyclohexanone as final products (26). The 19F NMR spectrum was recorded for the F-FMN-OYE in the presence of 5 equivalents of cyclohexenone. Interestingly, a single fluorine resonance was recorded at 85.3 ppm with a downfield shift of about 5 ppm. This is of the magnitude of the shift observed with the large molecule like testosterone which has also an α,β-unsaturated carbonyl moiety. Considering the fact that the binding of phenols perturbed the NMR spectrum negligibly, this might suggest that the protein binds phenolic and α,β-unsaturated carbonyl ligands in a different orientation. The absorption spectrum of the complex showed the formation of a charge transfer complex with the maximum located at 566 nm, due to the formation of phenol.

Determination of Redox Potential—The redox potential of 2'-F-FMN-OYE was determined by titration under anaerobic conditions with acetyl pyridine NADH and pyridine aldehyde NAD as described previously for native enzyme (30). Within experimental error the E₅₀ value for the overall two-electron potential, E₁/₂red, was the same as that measured for native enzyme (−230 ± 10 mV; results not shown). What is different, however, are the two separate one-electron potentials, E₁/₂EQ/Fₐq and E₁/₂EQ/Fₐred. With native enzyme, there is 15–20% thermodynamic stabilization of the anionic flavin semiquinone, in agreement with measured values for the E₁/₂EQ/Fₐq and E₁/₂EQ/Fₐred potentials of −245 mV and −215 mV, respectively (30). With 2'-F-FMN-OYE, no detectable amount of thermodynamically stable semiquinone could be observed. For example, during a xanthine/xanthine oxidase-catalyzed reduction of the enzyme in the presence of benzyl viologen as mediator (31), no semiquinone intermediate could be detected, although the 15–20% stabilization of semiquinone with native enzyme could be measured readily under the same conditions. Thus, the first electron potential, E₁/₂EQ/Fₐq, must be significantly lower than with native enzyme. With a limit of detection of 1% semiquinone, it can be calculated that the potential of the E₁/₂EQ/Fₐq couple would be 60 mV lower than that of the midpoint potential for the E₁/₂EQ/Fₐred couple, i.e. −290 mV. This value can be taken, therefore, as an approximate upper limit value for the first one-electron potential, and it is nicely consistent with the relationship previously found between the energy of the charge transfer transition with p-chlorophenol and the one-electron potentials of a series of artificial flavins bound to native Old Yellow Enzyme (30).

NMR and Ligand Binding Studies with the Individual Isozymes of OYE—OYE from brewers' yeast can be separated into three fractions by fast protein liquid chromatography: two homodimers and one heterodimer (6). Interestingly, the 2'-F-FMN-reconstituted wild type OYE, unlike the 8-F-FMN-reconstituted protein (4), showed only one fluorine resonance, suggesting similar chemical environments in this region of the active sites of the isozymes. To get more insight into this observation, two homodimers, fractions 1 and 3 of the OYE, were separated by fast protein liquid chromatography and subjected to further study. Apoproteins of these fractions were prepared and reconstituted with 2'-F-arabino-FMN. To find out if the flavin modification had affected the two isozymes differently, ligand binding and the 19F NMR of these reconstituted isozymes were studied. The 19F NMR of the 2'-F-arabino-FMN-reconstituted OYE fractions 1 and 3 showed one resonance at 80.3 ppm and 80.1 ppm, suggesting a similar chemical environment around the ribityl side chain. Titration of these two forms with 2,4-difluorophenol gave the Kᵅ value of 3–4 μM for fraction 1 and 1.5–2 μM for fraction 3. This again shows that the present chemical modification has a very similar effect on both the forms. The complex of fraction 1 has the resonances for the bound ligand at 32.7 and 30.8 ppm, whereas for fraction 3 they were recorded at 34.3 and 33.4 ppm (Fig. 4), suggesting that the interaction of ligand fluorines in both complexes are different. It can be concluded from the 19F NMR results with free and ligand-bound isozymes of OYE that noticeable differences in the chemical environments exist at the ligand binding site, whereas they are very similar around the ribityl side chain. When the reconstituted fractions were titrated with testosterone, the dissociation constants showed that both the isozymes bind this ligand with almost the same affinity.

Studies with 2'-Deoxy-FMN—In continuation of the above results with 2'-F-FMN-reconstituted OYE, it was thought appropriate to extend our studies to 2'-deoxy-FMN-reconstituted proteins. This flavin should act as a control for possible structural modifications induced by the fluorine substitution. The extinction coefficient for the 2'-deoxy-FMN was determined as 12,600 M⁻¹ cm⁻¹ by titration of the flavin with apoflavodoxin, which had been standardized with pure normal FMN.

2'-Deoxy-FMN-OYE—The binding of 2'-deoxy-FMN to
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Table III: Catalytic activities of Old Yellow enzyme substituted with modified flavins

| Observed turnover number | In the presence of Cyclohex-2-en-1-one | In the presence of 1,2-cyclohexanedione |
|-------------------------|----------------------------------------|----------------------------------------|
|                         | 200 μM min⁻¹ | 1 mM min⁻¹ | 200 μM min⁻¹ | 1 mM min⁻¹ |
| Wild type               | 32           | 150        | 150          | 85        |
| 2'-F-FMN-OYE            | 5.3          | 16.5       | 20           | 7.6       |
| 2'-deoxy-FMN-OYE        | 4.4          | 16         | 24           | 8.6       | 13.6 |

apoOYE was followed by measuring the flavin fluorescence. At the end point of the titration the fluorescence was quenched almost completely with a residual fluorescence of <2%. The \( \lambda_{max} \) of the free flavin shifted from 374 and 446 nm to 376 and 452 nm upon binding to the apoOYE. The dissociation constant was determined as 0.5 \( \times \) 10⁻⁸ M, intermediate between that for native FMN and 2'-F-FMN.

This form of the protein also forms a charge transfer complex with p-Cl-phenol with a maximum at 616 nm, shifted to lower wavelength by 32 nm, similar to what was observed with 2'-F-arabino-FMN-protein.

Activities of the Reconstituted Proteins—Old Yellow enzyme has NADPH oxidase activity but also reduces various \( \alpha,\beta \)-unsaturated ketones including guinones (6, 26). The catalytic activity for NADPH oxidation in the presence and absence of cyclohexenone and 1,2-cyclohexanediene was determined for modified proteins and found to be 10–15% of that of native enzyme. The results are documented in Table III.

The following conclusions can be made. 1) The binding affinity of the apoOYE with 2'-F-arabino-FMN was decreased substantially compared with that of normal FMN, probably due at least in part to disruption of two hydrogen bonds that the 2'-hydroxyl group makes with Pro-35 and Arg-243 (8).

2) The downfield shift of −15 ppm of the free flavin upon binding to apoOYE suggests strong protein-flavin interactions in this region. Although puckering at N-5 resulted in butterfly bending of the reduced flavin, the crystal structure showed no qualitative changes in the flavin-protein hydrogen bonding interactions (8). However, the C-5 atom of the FMN ribityl group moves on reduction by 1.6 Å to shift from one staggered conformation to another (8). Interestingly, the reduced 2'-F-arabino-FMN-OYE showed an upfield shift of 16 ppm, almost back to the free flavin chemical shift. This suggests that in the reduced enzyme the flavine might have lost the contact with protein as a result of the new conformation of the ribityl side chain.

3) With the 8-F-FMN-OYE, the 19F NMR spectrum for many of the complexes with various ligands showed two clearly distinguishable peaks (4). In contrast, with 2'-F-FMN-OYE the spectra of the various complexes showed clear single peaks. This suggests a similar chemical environment around the ribityl side chain for both isozymes.

4) The fact that the chemical shifts of the flavine from the protein-bound flavin showed no or negligible changes on complexation with phenols is consistent with the fact that their binding does not disrupt any interactions between the protein and flavin (8).

5) The fact that the flavine in the para-position of 2,4-difluorophenol and 4-F-phenol experiences a substantial upfield shift of −10 to −11 ppm suggests that the para-position of these ligands interact with Tyr-375. Since Tyr-375 lies in the active site and interacts strongly with the bound ligand, it will be of interest to further study its possible role in catalysis.

6) The fact that the binding of testosterone, which has an \( \alpha,\beta \)-unsaturated ketonic moiety was unaffected, while the binding of \( \beta \)-estradiol with a phenolic moiety suffered weak binding suggests that these two molecules orient differently at the active site on binding. Large chemical shifts observed for the cyclohexenone complex compared with the minor shifts on the binding of phenols are in accordance with this conclusion. These results are in support of the recent conclusion that the binding modes of cyclohexenone and 3-oxadecalin-4-one are different in the oxidative and reductive halves of the dismutation reaction catalyzed by OYE (26).

Glucose Oxidase—2'-F-arabino-FAD binds very tightly to apogluucose oxidase with quenching of the fluorescence and changes in the \( \lambda_{max} \) from 372 and 444 nm to 376 and 454 nm.

The 19F NMR spectrum of the 2'-fluoro-FAD-reconstituted protein showed a single peak at 66.38 ppm, again consistent with no major conformational changes upon reduction of the proteins (Tables I and II).

From enzyme-monitored turnover experiments (results not shown), the turnover number for the reconstituted enzyme was determined to be −5000 min⁻¹ at pH 7, 25 °C, which is 1/3 that of the native enzyme (−14,000 min⁻¹; Ref. 32).

\( \alpha \)-Amino Acid Oxidase—The absorption spectrum maxima for the 2'-F-arabino-FAD shifted from 372 and 444 nm to 362 and 448 nm upon binding to apoDAAO. The 19F NMR spectrum of the oxidized enzyme had a single resonance at 65.65 ppm, which is <1 ppm different from that of the free flavin, suggesting minor interaction of the fluorine with the protein. 2-aminolevulinic-acid-reduced protein showed a single resonance at 64.95 ppm, again consistent with no major conformational changes upon reduction. Oxidized \( \alpha \)-amino acid oxidase binds to benzoate, and as a result the absorption spectrum becomes highly resolved (33, 34). Similar spectral changes were observed in the case of modified protein. The 19F NMR spectrum of the complex had a resonance at 65.35 ppm, suggesting no significant changes in the protein-flavin interaction around the 2'-position of the ribityl side chain. The 19F NMR spectrum of the oxidized enzyme with sulfinic acid showed no major change in the fluorine chemical shift.

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