Absolute Stereochemistry of Flavins in Enzyme-Catalyzed Reactions†

Dietmar J. Manstein and Emil F. Pai*

Department of Biophysics, Max Planck Institute for Medical Research, D-6900 Heidelberg, Federal Republic of Germany

Lawrence M. Schopfer and Vincent Massey
Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

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ABSTRACT: The 8-demethyl-8-hydroxy-5-deaza-5-carba analogues of FMN and FAD have been synthesized. Several apoproteins of flavoenzymes were successfully reconstituted with these analogues. This and further tests established that these analogues could serve as general probes for flavin stereospecificity in enzyme-catalyzed reactions. The method used by us involved stereoselective introduction of label on one enzyme combined with transfer to and analysis on a second enzyme. Using as a reference glutathione reductase from human erythrocytes for which the absolute stereochemistry of catalysis is known from X-ray studies [Pai, E. F., & Schulz, G. E. (1983) J. Biol. Chem. 258, 1752-1758], we were able to determine the absolute stereospecificities of other flavoenzymes. We found that glutathione reductase (NADPH), general acyl-CoA dehydrogenase (acyl-CoA), mercuric reductase (NADPH), thioredoxin reductase (NADPH), p-hydroxybenzoate hydroxylase (NADPH), mellitolate hydroxylase (NADH), and glucose oxidase (glucose) all use the re face of the flavin ring when interacting with the substrates given in parentheses.

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FAD and FMN are ubiquitous coenzymes. They are extremely versatile redox catalysts, taking part in radical, carbonation, or hydride-transfer mechanisms (Hamilton, 1971; Hemmerich, 1976; Bruce, 1980; Walsh, 1980). They therefore occupy a central position in enzyme-catalyzed redox chemistry. At present, there are far more than 100 different flavoenzymes known, most of them members of the class of oxidoreductases (Enzyme Nomenclature, 1984).

Enzymatic (Jorns & Hersh, 1974; Fisher & Walsh, 1974; Hersh & Walsh, 1981; Thorpe & Williams, 1976) as well as biorganic model studies (Brustlein & Bruce, 1972; Loechler & Hollocher, 1980) have made it clear that positions C4α and N5 are the key loci of interaction between flavins and substrates. A common mechanism that has been proposed for many flavoenzymes involves the transfer of the equivalent of...
a hydride ion to N5 of the isoalloxazine ring system. As the two faces of the flavin ring are prochiral (Chart I), the question arose whether only one of them is used by any particular enzyme and if so which one.

The labile nature of the resulting N-H bonds made it impossible to use label transfer and from common flavin nucleotides to determine stereospecificities of flavoenzymes. In a first attempt to overcome this problem, 5-deaza-5-carba analogues of FAD and FMN were synthesized (O'Brien et al., 1970; Hersh & Jorns, 1975; Spencer et al., 1976) in order to make the prochiral center inert to solvent exchange. Further studies conclusively showed that there is direct hydrogen transfer from substrates to bound 5-deazaflavin1 coenzymes (Hersh & Jorns, 1975; Hersh et al., 1976; Jorns & Hersh, 1975, 1976; Fisher et al., 1976). Scrambling of label due to a rapid transfer from reduced to oxidized 5-deazaflavin molecules, however, did not allow determination of relative stereospecificities (Spencer et al., 1976).

Recently, it has been shown that analogous exchange between the oxidized and reduced forms of the riboflavin part of cofactor F20 from methanogenic bacteria (7,8-dide methyl-8-hydroxy-5-deazariboflavin) is several orders of magnitude slower than that of 5-deazariboflavin (Jacobson & Walsh, 1984). Taking advantage of this effect, first relative (Yamazaki et al., 1980) and later absolute stereochemistries of NAD+:FMN oxidoreductase from Beneckea harveyi and of several cofactor F20 dependent enzymes were determined (Yamazaki et al., 1985).

These findings made the use of 8-demethyl-8-hydroxy-5-deaza-5-carba-FAD and the corresponding FMN compound respectively the first choice when trying to develop a reasonably fast method of analyzing the absolute stereochemistry of flavin prosthetic groups. This analogue combines the redox properties of cofactor F20 with structural features as close as possible to those of native riboflavin nucleotides, permitting easier reconstitution of apoproteins with the analogue.

The first flavoenzyme for which the absolute stereochemistry of its prosthetic group became known was glutathione reductase from human erythrocytes. X-ray crystallography established that the nicotinamide ring of its substrate NADPH interacts with the re face of the isoalloxazine ring of its prosthetic group FAD (Pal & Schulz, 1983). On reducing 5-deaza-FAD-reconstituted general acyl-CoA dehydrogenase with NaB3H4, Ghisla et al. (1984) found that about 90% of tritium label had been incorporated into one side of the flavin ring. Combination of these results should allow determination of absolute stereospecificities by performing stereoselective labeling of the flavin analogue in general acyl-CoA dehydrogenase and reoxidizing it in glutathione reductase. Depending on whether the label would be released or whether it would stay at the flavin ring, general acyl-CoA dehydrogenase should use the re or si face, respectively. Then for any flavoenzyme to which the respective 8-OH-5-deaza cofactor can be bound and reduced or reoxidized by the corresponding substrate or substrate analogues, determination of absolute stereospecificity should be possible.

**Experimental Procedures**

**Materials**

5-Amino-o-cresol was obtained from TCI/Tokyo Kasei and 4-chlorouracil from Lancaster Synthesis. d-Ribose was from Sigma and trimethylthorformate from Aldrich. NAD (grade III from yeast), NADH (grade III from yeast), and Naja naja venom were purchased from Sigma. NADP+, NADPH, and 3-acetylpyridine adenine dinucleotides were obtained from P-L Biochemicals. Aquasolve scintillation fluid and NaB3H4 (lots 1749-181 and 1953-227; 8 mCi/mg) were from NEN. [1-3H]Glucose (25 Ci/mmol) was from ICN Chemical and Radioisotope Division and was used after dilution to 400 mCi/mmol. Ultrapure-grade guanidine hydrochloride was from Schwarz/Mann. All other chemicals were of the highest purity commercially available.

**Methods**

8-OH-5-deazariboflavins. 1-Deoxy-1-[(3-hydroxy-4-methylphenyl)amino]-d-ribitol was prepared by reacting 12.5 g of 5-amino-o-cresol with 15.5 g of d-ribose in 200 mL of absolute ethanol under nitrogen. The mixture was stirred at reflux for 1 h, then cooled down to room temperature, and diluted with another 150 mL of absolute ethanol. A total of 8 g of NaB3H4 was added in small portions to reduce the Schiff base. Then, the yellow golden solution was stirred for three more hours. The pH was adjusted to 5.0 with concentrated HCl, and 400 mL of well-degassed water was added. A total of 150 mL of AG 50W-X8 cation-exchange resin (100–200 mesh, H+ form) was brought into this solution, and the resulting slurry was gently stirred for 15 min. Further purification was performed at 4 °C in the dark. The mixture was layered on top of another 130 mL of ion-exchange resin packed into a column (2.5 x 54 cm). After being washed with 1.5 L of degassed water, the product was eluted with 1% ammonium hydroxide. Fractions containing the desired compound were combined and concentrated on a rotary evaporator at 40 °C to give 23 g (88% yield) of a light brown, amorphous material. This was further converted to 8-OH-5-deazariboflavin as published by Ashton and Brown (1980).

8-OH-5-deaza-FAD. 8-OH-5-deaza-FAD was prepared from 8-OH-5-deazariboflavin and ATP with riboflavin kinase/FAD synthetase partially purified from Brebibacterium ammoniagenes (Spencer et al., 1976; Manstein & Pai, 1986). About 80 mg of protein was dissolved in 150 mL of buffer containing 10 mM MES, 5 mM ATP, 30 mM MgCl2, 1 mM CaCl2, and 1 mM DTT. The pH was adjusted to 5.9 with 1 M KH2PO4. After addition of 12 mg of 8-OH-5-deazariboflavin, the mixture was incubated at 25 °C for 24–28 h. Progress of the conversion was monitored by HPLC (LKB...
modules) on a C-18 column (Abimed AnalySen-Technik GmbH, Shandon ODS Hypersil, 5 μm, 0.46 × 25 cm). Products of the FAD synthetase reaction were developed at a flow rate of 2.5 mL/min with a linear gradient from 90% solvent A (50 mM potassium phosphate, pH 6.0) to 45% solvent B (50 mM potassium phosphate buffer, pH 6.0, plus 50% CH₃CN) that took 6 min to reach the final conditions. The absorbance at 260 nm was followed.

After the reaction was completed, protein that had already been denatured was removed by centrifuging for 15 min at 18000g. The supernatant was filtered through an Amicon PM-10 membrane. The resulting clear solution was concentrated to approximately 15 mL on a rotary evaporator at 40 °C. It was then purified by applying 900-μL aliquots to a preparative HPLC column (Latek, Heidelberg; RP18, 10-μm HL, 1 × 25 cm). Elution was performed by a linear gradient at 5 mL/min. Solvent A was 50 mM triethylammonium acetate, pH 7.5; solvent B was 50 mM triethylammonium acetate, pH 7.5, plus 50% CH₃CN. The gradient ran from 0% to 45% of solvent B in 18 min.

8-OH-5-deaza-FMN. the FMN analogue was obtained from the modified FAD by hydrolysis with Nafta naja venom.

Enzymes and Apoenzymes. The following enzymes and corresponding apoenzymes were prepared as previously described: flavodoxin (Mayhew & Massey, 1969; Wassink & Mayhew, 1975) and α-lactate dehydrogenase (Olson & Massey, 1979) from Megaspernum elsdentii, L-lactate oxidase from Mycobacterium smegmatidis (Chong et al., 1975; Sullivan et al., 1977), glucose oxidase from Aspergillus niger (Swoboda & Massey, 1965; Swoboda, 1969), melittoid hydroxyline from Pseudomonas sp. (Strickland & Massey, 1973; Detmer et al., 1984), p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens (Enisch et al., 1970; 1980), d-amino acid oxidase from pig kidney (Brunby & Massey, 1966; Massey & Curti, 1966), and glutathione reductase from human erythrocytes (Krohne-Ehrich et al., 1977).

The following enzymes were provided as generous gifts: pig kidney general acyl-CoA dehydrogenase (Thorpe et al., 1979) by Dr. C. Thorpe (University of Delaware), apoenzymes of mercuric reductase from Pseudomonas aeruginosa PA 09501 carrying the plasmid pVSI (Fox & Walsh, 1982) and a mutant of the native enzyme with Cys-135 replaced by a serine (Schultz et al., 1986) by Dr. C. T. Walsh (Massachusetts Institute of Technology) and Dr. S. Miller (University of Michigan), and (from colleagues at the University of Michigan) an anthranilate hydroxylase from Trichosporum cutaneum (Powlowski & Dalley, 1982) by Dr. J. Powlowski, thioeoxdin reductase from Escherichia coli (O'Donnell & Williams, 1984) by Dr. C. H. Williams, Jr., and spinach ferredoxin-NADP⁺ reductase (Zanetti & Curti, 1980) by Dr. M. Ludwig. Spinach ferredoxin reductase apoenzyme was prepared as described by Zanetti et al. (1982). Aporproteins of an anthranilate hydroxylase and of general acyl-CoA dehydrogenase were resolved as described by Mayer and Thorpe (1981).

The apoprotein of thioeoxdin reductase was prepared by a slight modification of a procedure described by O'Donnell and Williams (1984). Native enzyme was incubated with 5 M guanidine in 0.1 M potassium phosphate–0.3 mM EDTA, pH 7.6, at 4 °C. One minute after addition of 1.65 mL of 8 M guanidine to 1 mL of a 50–100 μM solution of thioeoxdin reductase, this solution was transferred to a centrifuge tube containing a pellet of prewashed charcoal (Sigma Norit A) sufficient to make the resulting suspension 0.5% (dry weight/volume) in charcoal. The tube was centrifuged at 4 °C and 12800g for 3 min. The supernatant was transferred to another centrifuge tube containing prewashed charcoal. This step was repeated 3–4 times. Guanidine was finally removed by applying the solution to a Sephadex G-25 column equilibrated with 0.1 M potassium phosphate–0.3 mM EDTA, pH 7.6. Protein-containing factions were identified by measuring the optical absorbance at 280 nm.

The apoprotein of glutathione reductase from human erythrocytes was prepared by modifying a procedure described by Fritsch (1982). A total of 0.5 mL of 25–50 μM enzyme solution in 100 mM potassium phosphate, pH 7.0, 200 mM KCl, 1 mM EDTA, and 1.4 mM 2-mercaptoethanol cooled to 4 °C was mixed with 0.6 mL of 10 mM EDTA–1.4 mM 2-mercaptoethanol, pH 5.0, saturated with (NH₄)₂SO₄. To adjust the pH to 3.0, 55 μL of 1 M HCl was added. After being incubated on ice for 20 min, the apoprotein was pelleted in an Eppendorf centrifuge at 12800g for 6 min. The pellet was washed 4 times with 800 μL of 2.8 M (NH₄)₂SO₄, 10 mM EDTA, and 1.4 mM 2-mercaptoethanol, pH 3.0. Finally, the apoprotein was taken up in 150 μL of 100 mM Tris-HCl, pH 8.7, 10 mM EDTA, and 0.5 mM DTT.

Reconstitution of Apoenzymes with 8-OH-5-deazaflavins. Unless indicated otherwise, reconstitution of the respective apoenzymes with the 8-OH-5-deaza analogues was achieved by adding a 1.5–2 molar excess of flavin analogue to the apoenzyme dissolved in the appropriate buffer. The apoenzyme solutions used in this step were 20–60 μM. In order to remove surplus nucleotide and to exchange buffers, if necessary, the reaction mixtures were passed through Sephadex G-25 columns (1 × 25 cm).

Reconstitution of pig kidney general acyl-CoA dehydrogenase apoenzyme was performed by incubating a 20 μM solution of apoprotein in 50 mM potassium phosphate–0.3 mM EDTA, pH 7.6 (buffer A), with 1–2 equiv of 8-OH-5-deaza-FAD at 4 °C for 18 h. Surplus flavin was removed by adding this solution to a pellet of prewashed charcoal to yield a suspension 0.5% (weight/volume) in charcoal. After being incubated at 4 °C for 1 min, this solution was centrifuged at 12800g for 2 min. The supernatant was transferred to another centrifuge tube and the procedure repeated 2–3 times until the shoulder at 429 nm due to the visible absorption maximum of the free form of 8-OH-5-deazaflavin could no longer be detected. The peak of 8-OH-5-deaza-FAD bound to general acyl-CoA dehydrogenase (403 nm) does not interfere.

Test of Stereospecificity. General acyl-CoA dehydrogenase reconstituted with 8-OH-5-deaza-FAD was reduced by incubating 13 nmol of enzyme in 1 mL of buffer A with 0.5 mg NaB₃H₄ at 0 °C for 1 h. After this, no residual oxidized enzyme could be detected in absorption spectra of the reaction mixture. Residual borohydride was destroyed by adding 3 mg of sodium pyruvate before the solution was chromatographed over a G-25 fine column (1 × 20 cm) equilibrated with buffer A. Chiral 8-OH-5-deaza[5-H]FADH₂ was released by heating the protein to 100 °C for 1 min followed immediately by cooling in ice and centrifuging for 1 minute at top speed in an Eppendorf centrifuge at 4 °C.

Glucose oxidase was tested as an alternative way of stereoselectively labeling enzyme-bound 8-OH-5-deaza-FAD. A total of 10 nmol of reconstituted enzyme was incubated with 14 μmol of [1-3H]-d-glucose (0.05 mCi) at 25 °C. Progress of the reaction was followed spectrophotometrically. After reduction was complete, the solution was passed over a Sephadex G-25 fine column (1 × 20 cm). A total of 9.5 nmol of 8-OH-5-deaza[5-H]FADH₂ enzyme with a specific activity of 27 000 cpm/nmol was eluted from the column. The labeled flavin analogue was released by heating the enzyme to 100 °C.
Analogues. The determination of the stereochemistry of the reaction products was achieved by adding the appropriate nicotinamide nucleotide and followed spectrophotometrically. After completion of the reaction, an aliquot of the solution was passed through a Sephadex G-25 column with a void volume of 5 mL. Each fraction was analyzed for radioactivity, and its absorption spectrum was recorded. In order to concentrate most data relevant to a specific enzyme, details for individual enzymes will be given under Results.

Instrumentation. Optical spectra were recorded on Cary 219 or Shimadzu UV-260 spectrophotometers. Fluorescence spectra were measured on a ratiomax recording fluorometer built by Dr. D. Ballou and G. Ford of the University of Michigan. Radioactivity was determined with a Beckman LS 7800 liquid scintillation counter. Selected vials were calibrated internally. The average counting efficiency was about 40%. HPLC was performed on an LKB instrument equipped with a Model 2150 pump, UV detector 2151, solvent programmer 2152, and a Shimadzu C-R1a integrator. Stopped-flow kinetics were measured with a temperature-controlled instrument interfaced with a Nova 2 (Data General) minicomputer system (Beatty & Ballou, 1981).

Results

Synthesis of 8-OH-5-deazaflavins. When the Schiff base formed upon condensation of 5-amino-o-cresol with d-ribose was reduced with NaBH₄, 5-deoxy-1-(3-hydroxy-4-methylphenylamino)-d-ribofuranose was obtained in pure form and in good yield. Conversion of this compound to 8-OH-5-deazariboflavin according to Ashton and Brown (1980) gave 321 mg of golden yellow product.

By modifying the procedure for the purification of the FAD-synthesizing activity from Brevibacterium ammoniagenes, we were able to obtain an enzyme preparation of increased stability that allowed complete conversion of 8-OH-5-deazariboflavin to the FAD level (Manstein & Pai, 1986). The purity of the product was >95% as judged by HPLC analysis.

In trying to improve the yield of the enzymatic reaction, we found that pH is an important variable to optimize. In the conversion of 8-OH-5-deazariboflavin, maximal activity was obtained at pH 5.9. Below pH 5.8, denaturation of the enzyme started to be a problem; above this pH, the rate of the enzymatic reaction rapidly decreased, consistent with only the neutral forms of flavins being accepted as substrates. Curiously, addition of 1 mM CaCl₂ to the reaction mixture was found to have a pronounced stabilizing effect on the enzyme (Manstein & Pai, 1986).

Chemical and Spectral Properties of 8-OH-5-deazaflavins Analogue. The redox properties of 8-OH-5-deazariboflavin were found to be virtually identical with those of 7,8-dide methy1-8-OH-5-deazariboflavin described by Jacobson and Walsh (1984). A spectrophotometric titration (Figure 1) gave a pKₐ of 6.1 for both 8-OH-5-deaza-FMN and 8-OH-5-deazariboflavin. A pKₐ of 6.0 was found.

When 8-OH-5-deaza-FAD at pH 7.6 was treated with Naja naja venom, the maximum of the visible absorption peak shifted from 430 to 426 nm (Table 1). Its intensity increased by 8%, an amount comparable with the value of 11% observed on hydrolysis of FAD to FMN (Bessey et al., 1949).

For the native coenzymes, these changes are accompanied by an about 10-fold increase in fluorescence. This can be explained by release of self-quenching due to intramolecular interactions between the planar purine and isoalloxazine systems (Weber, 1950). A similar self-quenching was not observed with 8-OH-5-deaza-FAD at neutral pH, suggesting that this kind of "hairpin" complexation can only occur between uncharged subunits. Ghisla and Mayhew (1980) obtained similar results investigating 8-demethyl-8-OH-FAD.

Further support comes from the fact that in both the FMN and the FAD forms the pKₐ of the 8-hydroxy substituent is nearly identical, which again is not expected if the purine and isoalloxazine rings interact. Upon reduction to the dihydro form, fluorescence was almost completely lost.

Reconstitution Experiments and Results of Stereospecificity Tests. With the exception of Old Yellow Enzyme, all flavoenzymes tested so far showed tight binding of the respective oxidized 8-OH-5-deazaflavin cofactor. No loss of analogue was observed upon gel filtration, and the spectral features remained constant (Table II). This included the apoproteins of d-lactate dehydrogenase (EC 1.1.99.6), d-amino acid oxidase (EC 1.4.3.3), and ferredoxin-NAD⁺ reductase (EC 1.18.1.2) as FAD enzymes and L-lactate oxidase (1.1.3.12.4) and flavodoxin as FMN enzymes beside those flavoenzymes for which the stereochemistry has been elucidated, and more details are given below. With some of these proteins, association with the reduced form of the analogue has been tested.
such a behavior has also been described for the rebinding of the dehydrogenase, 8-OH-5-deaza-FAD (pKₐ produced the bound flavin stereoselectively (Ghisla et al., 1984), with enzyme reconstituted with 5-deaza-FAD, NaB₃H₄ re-

4.8 for free 8-OH-FAD (Ghisla et al., 1979), respectively. The addition of octanoyl-CoA (20 μM) or acetocetyl-CoA (50 μM) perturbed the oxidized flavin chromophore, leading to an intensification and 5-nm red shift of the absorbance peak. Neither reduction nor long wavelength band formation was detectable upon addition of the thio ester substrates. This reflects the considerably more negative oxidation–reduction potential of 8-OH-5-deaza-FAD compared to FAD.

In contrast to enzyme reconstituted with 8-OH-FAD or 8-SH-FAD, which shows a marked tendency to lose its respective chromophore (Thorpe & Massey, 1983), the 8-OH-5-deaza-FAD form of general acyl-CoA dehydrogenase was stable and could be stored at 4 °C for at least 1 month. This is consistent with the finding that the apoenzyme preferentially binds neutral isoalloxazine species. While the pKₐ values of 4.8 for free 8-OH-FAD (Ghisla & Mayhew, 1976) and 3.8 for free 8-SH-FAD (Moore et al., 1979), respectively, are too low to be elevated into the neutral pH range upon binding to the dehydrogenase, 8-OH-5-deaza-FAD (pKₐ = 6.1; Figure 1) can be found in its neutral form.

Reconstituted enzyme was reduced as described above. The resulting spectrum is also given in Figure 2. As was the case with enzyme reconstituted with 5-deaza-FAD, NaB₃H₄ reduced the bound flavin stereoselectively (Ghisla et al., 1984), introducing the tritium label at the re side of the flavin ring, as will be documented below.

**Glutathione Reductase (EC 1.6.4.2).** When 8-OH-5-deaza-FAD was incubated with the apoprotein of glutathione reductase from human erythrocytes, the reconstitution reaction was clearly biphasic. Rapid initial attachment of the flavin to the protein moiety was followed by a slow rearrangement. Such a behavior has also been described for the rebinding of FAD to the apoprotein (Staal et al., 1969). Spectral data for the species obtained after incubation for 1 h are given in Table II. The reconstituted enzyme had no detectable reductase activity, which is consistent with the results of Chan and Bruce (1977), who reported that 5-deazaflavins cannot transfer electrons to thiols, and also with the finding of Krauth-Siegel et al. (1985) that glutathione reductase when reconstituted with 5-deaza-FAD has no reductase activity anymore. However, the addition of a 4-fold excess of NAD⁺ to a solution containing reduced 8-OH-5-deaza enzyme led to the complete reoxidation of the flavin chromophore within the time of mixing. One equivalent of NADPH reduced about 50% of the enzyme-bound 8-OH-5-deaza-FAD. Excess reductant did not significantly increase the amount of reduced enzyme. A very similar result was obtained when glutathione reductase reconstituted with 5-deaza-FAD was titrated with NADPH (R. L. Krauth-Siegel, S. Ghisla, and E. F. Pai, unpublished results). The fact that the reduced form of the analogue binds tightly to the apoenzyme and the competence of 8-OH-5-deazaglutathione reductase in catalyzing transhydrogenation are of major importance since glutathione reductase is the only flavoenzyme for which the stereochemistry of catalysis has been independently established with the native enzyme (Pai & Schulz, 1983). This knowledge is the basis for all interpretations of the results described below.

![Absorbance](image-url)  
**FIGURE 2:** Optical absorption spectra of 14.5 μM pig kidney general acyl-CoA dehydrogenase reconstituted with oxidized (—) or NaB₃H₄-reduced (—) 8-OH-5-deaza-FAD in 50 mM phosphate buffer-0.3 mM EDTA, pH 7.6, at 4 °C.

<table>
<thead>
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<th>protein</th>
<th>source</th>
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<th>εₘₐₓ (cm⁻¹ M⁻¹)</th>
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<td><em>P. aeruginosa</em></td>
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<td>nd</td>
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<td>430</td>
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*In buffer A as solvent. **Single-site mutant with Cys-135 replaced by Ser-135.*
of NAD+, no further changes in the visible spectrum of the apoprotein at 20 nmol of the labeled analogue was incubated with 18 nmol of Ser-135 mutant enzyme of mercuric reductase. A total of 5 containing protein.

A total of 84% of the counts coeluted with the protein; 93% was found in the small molecule fractions. Reconstitution of the apoenzyme with 8-OH-5-deaza-FAD yielded the spectrum of the fully reduced chromophore. Contrary to what had been found with glutathione reductase and mercuric reductase, the intensity of the visible absorption peak of the flavin chromophore came back to only ~80% when the reduced form of the enzyme was reoxidized by addition of NAD+. This was accompanied by a shift of the maximum from 414 nm to 396 nm with a shoulder at 414 nm still observable. However, when this enzyme was denatured, the flavin released was completely oxidized; therefore, the spectral differences should not be due to incomplete reoxidation. In order to check whether the denaturation procedure caused further oxidation of the chromophore, the same kind of treatment was performed with fully reduced enzyme. No reoxidation of the flavin analogue could be observed. When AcPyADP+ was used instead of NAD+, reoxidation was much faster, but the resulting spectrum still appeared as described above.

After 5 nmol of chiral 8-OH-5-deaza[5-3H]FADH2 in buffer A had been incubated with a 5-fold excess of thioredoxin reductase apoprotein at 4 °C for 10 min, 200 nmol of AcPyADP+ was added to reoxidize the reconstituted enzyme. A 300-μL aliquot was passed through the G-25 fine column, and 900-μL fractions were collected. A total of 9% of the radioactivity was found to coelute with the protein, 91% with the pyridine nucleotides.

**Glucose Oxidase (EC 1.1.3.4).** At 25 °C it took 20 min until the apoprotein of *A. niger* glucose oxidase had completely combined with 8-OH-5-deaza-FAD. The reaction could be followed spectrophotometrically when an excess of apoprotein was added. As with the 5-deaza-FAD-reconstituted enzyme (Fishel et al., 1976), addition of D-glucose caused rapid bleaching of the flavin peak. The spectra indicated that recombination of the bound flavin went nearly to completion (Figure 4). In further analogy of the 5-deaza-FAD enzyme, no oxygen was consumed when D-glucose was used to reduce the reconstituted enzyme, indicating that reduction was stoichiometric, not catalytic. When glucose oxidase was reconstituted with reduced 8-OH-5-deaza-FAD and passed through a Sephadex G-25 column, no reoxidation could be detected. Even after addition of 2 equiv of gluconolactone, the enzyme stayed in its reduced form.

In order to describe the kinetics of the reduction of 8-OH-5-deaza-FAD-reconstituted glucose oxidase by substrate in more detail, stopped-flow experiments were performed (Figure 4), measuring the changes of absorbance at 410, 350, and 320 nm. All three wavelengths gave the same results. The rate of reduction was proportional to D-glucose concentration up to the highest concentrations that could be used. The corresponding second-order rate constant was 24.7 M⁻¹ s⁻¹. Also with the native glucose oxidase a similar lack of evidence for the formation of a Michaelis complex in the first step of the reductive half-reaction has been reported. When the now stereospecifically labeled flavin was transferred to 30
nmol of mercuric reductase apoprotein and stereospecificity was analyzed as described above with 100 nmol of NAD+ for reoxidation, only 7% of the tritium was found in the protein peak, the rest being associated with small molecules.

**p-Hydroxybenzoate Hydroxylase (EC 1.14.13.2).** 8-OH-5-deaza-FAD was rapidly bound by the apoprotein of p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*. The absorption maximum was shifted to 435 nm. Addition of p-hydroxybenzoate shifted it further to 443 nm. Addition of D-glucose to a final concentration of 35 mM led to the formation of spectrum (——). The inset shows a plot of the rate of the half-reaction, $E_{ox} + \text{glucose} \rightarrow E_{red}P$, as determined by mixing 8-OH-5-deaza-FAD-reconstituted glucose oxidase with D-glucose in the stopped-flow apparatus.

A total of 2.5 nmol of stereoselectively tritiated analogue was incubated with 16 nmol of p-hydroxybenzoate hydroxylase apoprotein at 4°C for 5 min. Reoxidation was started by simultaneously adding 200 nmol of AcPyADP+ and 100 nmol of p-hydroxybenzoate. After 20 min, there were no further changes in the absorption spectrum. Preliminary experiments had indicated that reduced AcPyADP might form a tight complex with the enzyme, which would interfere with analysis of stereospecificity. To displace labeled pyridine nucleotide from this complex, another 500 nmol of AcPyADP+ was added; 400 μL of this solution was applied to a gel filtration column, and 770-μL fractions were collected. Only 5% of the radioactivity was found to coelute with the protein.

In a control experiment, 5 nmol of the labeled flavin were bound to p-hydroxybenzoate hydroxylase apoprotein as described above, but this time 600 μL of the solution containing reconstituted enzyme was passed directly through a gel filtration column. A total of 90% of the label was found to coelute with the enzyme. Now fractions containing most of the protein were pooled and concentrated. p-Hydroxybenzoate and AcPyADP+ were added to reoxidize the analogue. The resulting solution was applied to another gel filtration column. After reoxidation, only 14% of the label was still associated with the protein while the rest was found to coelute with AcPyADP+.

**Melilotate Hydroxylase (EC 1.14.13.4).** Reduced 8-OH-5-deaza-FAD bound to p-hydroxybenzoate hydroxylase before addition of AcPyADP+ (0 min) and after complete reoxidation of the enzyme-bound analogue (20 min), respectively.

In an inset shows a plot of the rate of the half-reaction, $E_{ox} + \text{melilotate} \rightarrow E_{red}P$, as determined by mixing 8-OH-5-deaza-FAD-reconstituted p-hydroxybenzoate hydroxylase before addition of AcPyADP+ (0 min) and after complete reoxidation of the enzyme-bound analogue (20 min), respectively.

The absorption maximum was shifted to 435 nm. Addition of melilotate and of its analogue salicylate perturbs absorption spectrum. Preliminary experiments had indicated that reduced AcPyADP might form a tight complex with the enzyme, which would interfere with analysis of stereospecificity. To displace labeled pyridine nucleotide from this complex, another 500 nmol of AcPyADP+ was added; 400 μL of this solution was applied to a gel filtration column, and 770-μL fractions were collected. Only 5% of the radioactivity was found to coelute with the protein.

In a control experiment, 5 nmol of the labeled flavin were bound to p-hydroxybenzoate hydroxylase apoprotein as described above, but this time 600 μL of the solution containing reconstituted enzyme was passed directly through a gel filtration column. A total of 90% of the label was found to coelute with the enzyme. Now fractions containing most of the protein were pooled and concentrated. p-Hydroxybenzoate and AcPyADP+ were added to reoxidize the analogue. The resulting solution was applied to another gel filtration column. After reoxidation, only 14% of the label was still associated with the protein while the rest was found to coelute with AcPyADP+.

**Anthranilate Hydroxylase (EC 1.14.12.2).** Addition of the substrate anthranilate and of its analogue salicylate perturbs the oxidized flavin chromophore leading to an increase in absorbance,

$\epsilon_{250} = 42400 \text{ M}^{-1} \text{ cm}^{-1}$ in the presence of anthranilate and

$\epsilon_{250} = 45500 \text{ M}^{-1} \text{ cm}^{-1}$ in the presence of salicylate, respectively. When the reduced enzyme was incubated with 2.7 mM AcPyADP+ at 10°C in the presence of substrate, slow reoxidation of the enzyme-bound flavin could be obtained.

A total of 18 nmol of anthranilate hydroxylase apoenzyme was incubated at 10°C in the presence of 9 nmol of stereoselectively tritated analogue and 200 nmol of anthranilic acid. After 5 min, two aliquots of 500 μL each were taken. One of these aliquots was immediately applied to gel filtration; the other had 1.5 μmol of AcPyADP+ added. When the reduced enzyme was passed through the gel filtration column, 96% of the radioactivity was found to elute under the enzyme peak. Reoxidation in the presence of AcPyADP+ proceeded very slowly; at 10°C at least 70 min were needed until full reox-
Identification of the enzyme-bound analogue was achieved. After reoxidation, 76% of the counts were found in fractions containing the pyridine nucleotides. The rather slow rate of reaction may be the reason why with this enzyme more counts than usual are found in the protein fraction of the gel filtration column.

In Table III the distribution of radioactive counts between fractions containing protein and fractions containing small molecules is given for all flavoenzymes tested so far.

### DISCUSSION

It is obvious that a flavin analogue suitable for probing stereospecificities of flavin cofactors in enzymic reactions has to have several specific features: its shape and charge should not interfere with correct binding at the enzyme's active site; bonds formed upon labeling should be stable against solvent exchange; there should also be no scrambling of the kind found with 5-deazaflavins (Spencer et al., 1976); reoxidation by oxygen should be slow to make handling easier; the riboflavin form of the analogue should be a substrate of one of the known riboflavin kinase/FAD synthetase systems to enable facile production of the corresponding FMN and FAD forms. With the exception of the last feature, the hydrolysis products of cofactor F<sub>420</sub> 7,8-didemethyl-8-hydroxy-5-deazariboflavin and the corresponding FMN form, meet these requirements. Consequently, they were used in experiments to determine the stereospecificities of a couple of F<sub>420</sub>-dependent enzymes from methanogens. The oxidoreductase from *Beneckea harveyi* could be included, as this enzyme also accepts riboflavins as substrates (Yamazaki et al., 1980, 1985). However, 7,8-didemethyl-8-hydroxy-5-deazariboflavin is one of the few analogues that is not turned over by the riboflavin kinase/FAD synthetase from *Brevibacterium ammoniagenes*, the best known and most widely used tool for transforming riboflavins to the corresponding FMN or FAD forms. With the spectra of the enzymes reconstituted with the respective analogues show considerable variations in wavelengths and extinction coefficients of their absorption maxima (Table II).

Glucose oxidase and general acyl-CoA dehydrogenase clearly stabilize the neutral state of 8-OH-5-deazaflavins with absorption maxima shifted to lower wavelengths and strongly reduced extinction coefficients (Figure 1). On the other side, the hydroxylases tested bind the anionic analogue, leading to red-shifted peaks with less pronounced decrease in absorption. Interpretation of the absorption spectrum of glutathione reductase is not so straightforward as it combines long-wavelength absorption (434 nm) with a relatively low extinction coefficient. Taking into account also the shape of the curve together with structural information on the environment of the flavin binding site (Schulz et al., 1982), it seems more likely that in this case the anionic form is bound.

Besides the availability of an analogue combining all the advantages mentioned above, there were two published results that were also crucial for the success of the work described in this paper. The finding of Ghislé et al. (1984) that 5-deazaflavin can be stereoselectively labeled by NaB<sub>3</sub>H<sub>4</sub> when bound to pig kidney general acyl-CoA dehydrogenase provided a very convenient way of introducing a radioactive marker. In addition, during the course of the present work another route, transferring tritium from [1-<sup>3</sup>H]glucose to the flavin, was also tried and found to be equally useful. The second anchor point was the elucidation of the absolute stereochemistry of the catalytic mechanism of glutathione reductase from human erythrocytes by means of X-ray crystallography (Pai & Schulz, 1983). So, this enzyme could be used as reference for elucidating absolute stereospecificities of flavins involved in enzymic reactions.

After stereoselective reduction of 8-OH-5-deaza-FAD in general acyl-CoA dehydrogenase, release of the analogue was achieved by heat denaturation. By keeping the time of exposure to high temperature rather short (~1 min), immediately followed by cooling on ice where the free 8-OH-5-dea-

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**Table III: Determination of Chirality of 8-OH-5-deaza[<sup>5</sup>H<sub>2</sub>]FADH<sub>2</sub>**

<table>
<thead>
<tr>
<th>enzyme used for labeling</th>
<th>glutathione reductase</th>
<th>mercuroc reductase&lt;sup&gt;e&lt;/sup&gt;</th>
<th>mercuroc reductase&lt;sup&gt;d&lt;/sup&gt;</th>
<th>p-hydroxybenzoate hydroxylase</th>
<th>mercuroc reductase&lt;sup&gt;e&lt;/sup&gt;</th>
<th>mercuroc reductase&lt;sup&gt;d&lt;/sup&gt;</th>
<th>thioredoxin reductase</th>
<th>mercuroc reductase&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>gen acyl-CoA dehydrogenase</td>
<td>39.5 (80)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>9.9 (20)</td>
<td>36.0 (90)</td>
<td>4.0 (10)</td>
<td>38.7 (90)</td>
<td>4.3 (10)</td>
<td>6.4 (9)</td>
<td>14.5 (7)</td>
</tr>
<tr>
<td>gen acyl-CoA dehydrogenase</td>
<td>4.2 (9)</td>
<td>43.0 (91)</td>
<td>16.1 (16)</td>
<td>84.0 (84)</td>
<td>2.0 (7)</td>
<td>27.0 (93)</td>
<td>3.3 (9)</td>
<td>33.7 (91)</td>
</tr>
<tr>
<td>gen acyl-CoA dehydrogenase</td>
<td>13.7 (24)</td>
<td>43.3 (76)</td>
<td>13.7 (24)</td>
<td>43.3 (76)</td>
<td>6.4 (9)</td>
<td>63.7 (91)</td>
<td>14.5 (7)</td>
<td>192.5 (93)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Performed as described under Methods. <sup>b</sup>After gel filtration. <sup>c</sup>Values in parentheses are percentages. <sup>d</sup>Mutant enzyme with Cys-135 replaced by Ser-135. <sup>e</sup>[1-<sup>3</sup>H]Glucose was used to reduce enzyme-bound 8-OH-5-deaza-FAD.
analyses to these classes of enzymes, too. The analogue might not be straightforward. As indicated at the end of Results, we are presently trying to extend our method described in this paper is not intended to test mechanistic proposals but was developed to determine which side of the prochiral ring of the prosthetic group of a flavoenzyme interacts with a given substrate. Therefore, it is quite conceivable that this method could also be applied to enzymes that use a carbanion mechanism or work by handling single electrons at a time, like flavodoxin. It may, however, be more tedious to use this method on such proteins, as answering the question what should be used for reduction or reoxidation of the analogue might not be straightforward. As indicated at the end of Results, we are presently trying to extend our analyses to these classes of enzymes, too.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>source</th>
<th>substrate</th>
<th>side of flavin ring interacting with substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutathione reductase</td>
<td>human erythrocytes</td>
<td>NADPH</td>
<td>re&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>general acyl-CoA dehydrogenase</td>
<td>pig kidney</td>
<td>acyl-CoA, NaBH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>re</td>
</tr>
<tr>
<td>mercuric reductase</td>
<td>P. aeruginosa</td>
<td>NADPH</td>
<td>re</td>
</tr>
<tr>
<td>thioredoxin reductase</td>
<td>E. coli</td>
<td>NADPH</td>
<td>re</td>
</tr>
<tr>
<td>glucose oxidase</td>
<td>A. niger</td>
<td>glucose</td>
<td>re</td>
</tr>
<tr>
<td>p-hydroxybenzoate hydroxylase</td>
<td>P. fluorescens</td>
<td>NADPH</td>
<td>re</td>
</tr>
<tr>
<td>melilotate hydroxylase</td>
<td>P. sp.</td>
<td>NADH</td>
<td>re</td>
</tr>
<tr>
<td>anthranilate hydroxylase</td>
<td>T. cutaneum</td>
<td>NADPH</td>
<td>re</td>
</tr>
</tbody>
</table>

<sup>d</sup>From Pai and Schulz (1983). <sup>e</sup>See Ghisla et al. (1984).

azaflavin was trapped with apoprotein, it was possible to avoid problems of reoxidation or label scrambling. When enzymes reconstituted with the reduced analogues were subjected to gel filtration, there was no loss of flavin and the label was found in the protein fraction. After reoxidation by substrates or substrate analogues, most of the counts, usually around 90%, appeared in the small molecule peak of an additional gel filtration (Table III). Oxygen does not play a significant role in reoxidation of reduced 8-OH-5-deazaflavins, as has been shown above. Furthermore, unspecific reoxidation should lead to preferential loss of hydrogen leaving the tritium label at the face of the flavin ring with the substrates used.

**References**


Hemmerich, P. (1976) in Progress in the Chemistry of Organic Natural Products (Herz, W., Grisebach, H., & Kirby, G.)


