Wallace, R. W., Tallant, E. A., & Cheung, W. Y. (1980b) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. 1, pp 14–40, Academic Press, New York.
Walsh, M. P., Dabrowska, R., Hinkins, S., & Hartshorne, D. J. (1982) *Biochemistry 21*, 1919–1925.
Wang, J. H., Sharma, R. K., & Tam, S. W. (1980) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. 1, pp 305–328, Academic Press, New York.
Wood, J. G., Wallace, R. W., Whitaker, J. N., & Cheung, W. Y. (1980) *J. Cell Biol. 84*, 66–76.
Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., & Uenishi, K. (1978) *J. Biol. Chem. 253*, 1388–1390.
Yang, S.-D., Tallant, E. A., & Cheung, W. Y. (1982) *Biochem. Biophys. Res. Commun. 106*, 1419–1425.

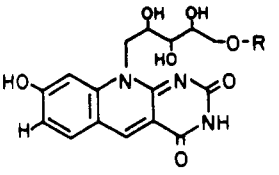
Properties of 7,8-Didemethyl-8-hydroxy-5-deazaflavins Relevant to Redox Coenzyme Function in Methanogen Metabolism†

Fredric Jacobson‡ and Christopher Walsh*

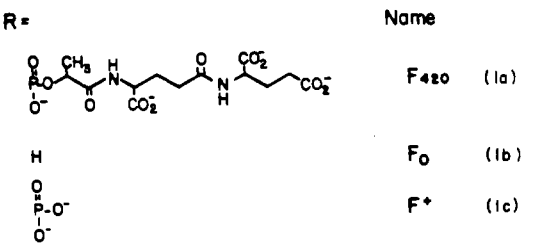
ABSTRACT: The 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) moiety is the key element in the newly discovered redox coenzyme factor 420 (F420) found in methanogenic bacteria and in streptomycetes. In this paper, we have analyzed chemical properties of synthetic FO that condition coenzyme function and compare FO to 5-deazariboflavin (5-dRF) and 8-hydroxyriboflavin. The equilibrium constants for sulfite addition and the rates of reoxidation of FOH_2 by a series of flavin analogues show that the 5-carba substitution imposes nicotinamide-like chemistry on the system, including sluggishness to reoxidation by O_2. Ionization of the 8-OH substituent in the oxidized FO (pK_a = 5.85) suppresses reactivity of FO toward redox chemistry. In the reduced FOH_2, the phenolic group is isolated and shows a more normal pK_a of 9.7. The reduction potential of FO/FOH_2 has been determined by equilibration with two methanogen enzymes, an F420-reducing hydrogenase and an NADP:F420 oxidoreductase, to be −340 to −350 mV. The rate of the bimolecular disproportionation of FOH_2 and FO was followed by high-pressure liquid chromatography analysis, starting with tritium in the oxidized species, and shown to be 10–20 M^{-1} min^{-1}, down 50–100-fold from the 5-dRF/5-$dRFH_2$ reaction. This extended lifetime of chiral [5-^{3}H]FOH_2 samples in the presence of FO molecules permits stereochemical determination of hydride transfers to and from C(5) of the 8-hydroxy-5-deazaflavin system. Methanogen hydrogenase and NADP:F420 oxidoreductase are defined to show "A" side specificity while the NAD:FMN oxidoreductase from *Benekea harveyii* shows "B" side specificity.

In addition to the characteristics that distinguish all Archaebacteria from other Procaryotae (Eubacteria) (Balch et al., 1977, 1979; Woese et al., 1978), the methane-producing bacteria possess a variety of apparently unique cofactors involved in electron and one-carbon transport. These include the nickel tetrapyrrole F430 (Gunsalus & Wolfe, 1978; Whitman & Wolfe, 1980; Diekert et al., 1980a–c), coenzyme M (2-mercaptoethanesulfonate) (Taylor & Wolfe, 1974), F342 (Gunsalus & Wolfe, 1978), and the yellow fluorescent cofactor (YFC) (Daniels, 1978). Methanogens also contain the redox-active cofactor F420[1] (I) (Cheeseman et al., 1972; Eirich et al., 1979), which has been identified as a derivative of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (II) (Eirich et al., 1978; Ashton et al., 1979). This molecule is used to link H_2 or formate oxidation to reduction of NADP (Tzeng et al., 1975a,b) and perhaps to serve as reductant in the last step of methane biosynthesis (Ellefson & Wolfe, 1980, 1981).

This natural product 5-deazaflavin is not restricted to the methanogens. Eker and his co-workers have recently found the chromophore in cell extracts of *Streptomyces griseus* and in a homogeneous preparation of its thymine dimer cleaving photoreactivation enzyme (PRE) (Eker et al., 1980, 1981; Eker, 1980). As long ago as 1960, McCormick and co-workers described a "cosynthase" factor, with the same spectroscopic features as F420, involved in chlortetracycline biosynthesis by another *Streptomyces* strain (McCormick et al., 1960; Miller et al., 1960) and have now assigned to it an 8-hydroxy-5-deazaflavin structure (McCormick & Morton, 1982). Prelim-



7,8-didemethyl-8-hydroxy-5-deazariboflavin (oxidized)



† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received July 8, 1983.* Supported in part by Grant GM 21643 from the National Institutes of Health and research funds from Merck Sharp & Dohme, Inc.

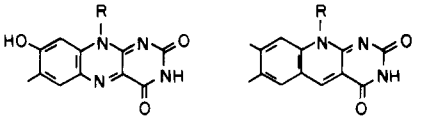
‡ Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

[1] Abbreviations: FO, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; F^+, 5′-phosphate of FO; F420, the natural product methanogen deazaflavin cofactor; RF, riboflavin; 5-dRF, 5-deazariboflavin; 8-OH-5-dRF, 8-demethyl-8-hydroxy-5-deazariboflavin; 8-OH-5-dFl_{Et}, *N*(10)-ethyl-8-hydroxy-5-deazaisoalloxazine; 8-MeO-5-dFl_{Et}, *N*(10)-ethyl-8-methoxy-5-deazaisoalloxazine; H_2ase, hydrogenase; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; MES, 4-morpholineethanesulfonic acid; HPLC, high-pressure liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid. In FOH_2 and 5-$dRFH_2$, the H_2 suffix denotes that the flavin is in the 1,5-dihydro form.

0006-2960/84/0423-0979$01.50/0

inary results (Eker et al., 1981) further suggest the presence of F420 in the photoreactivation enzymes of *Anacystis nidulans* and *Agmenellum quadruplicatum*, and it is likely that the coenzyme will be detected in still other microorganisms.

Given its important role in methanogen redox metabolism and the evidence of its more widespread distribution, we have undertaken an investigation of the redox properties, chemical reactivity, and enzymatic processing of this novel flavin coenzyme. Prior studies of 8-hydroxyflavins (III) and 5-deazaflavins (IV) in this and other laboratories provide a benchmark for understanding the properties of F420, a structural hybrid of these two flavin analogues. We have performed experiments with 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) rather than with F420 itself because of the larger quantity of the FO available to us by a synthetic route (Ashton et al., 1979; Ashton & Brown, 1980). In enzymatic experiments, no significant distinctions in behavior have yet been observed between F420 and the simpler FO moiety (Eirich et al., 1979; Jacobson et al., 1982; Yamazaki et al., 1980).



III IV

Experimental Procedures

Chemicals

Riboflavin analogues, including FO, were the gift of Drs. W. Ashton, R. Brown, and E. Rogers of Merck Sharp & Dohme Research Laboratories. *N*(10)-Ethyl-8-hydroxy-5-deazaisoalloxazine (8-OH-5-dFl$_{Et}$) and its 8-methylated form (8-MeO-5-dFl$_{Et}$) were the gift of Dr. L. Tsai of the National Institutes of Health (NHLBI). F^+, the 5′-phosphate of FO, was produced both by acid hydrolysis of methanogen F420 (Eirich et al., 1978) and by chemical phosphorylation of FO (Scola-Nagelsheider & Hemmerich, 1976). In both cases, the product was purified as described in Eirich et al. (1978). Fluorometric titration with apoflavodoxin indicated that only 85% of the F^+ was bound, suggesting that the remainder was the 4′-isomer (Mayhew et al., 1974). Diquat (DQ) was provided by Dr. W. H. Orme-Johnson. All other chemicals, excluding those mentioned below, were obtained from commercial sources and used without further purification.

[5-^{3}H]FO. FO (1–2 mg/mL) in aerobic Na_2CO_3 (1 mg/mL) was reduced, at 40 °C, with an excess of NaB^3H_4 (100 mCi/mmol, New England Nuclear, Boston, MA). Reaction was monitored by measuring the 420-nm absorbance of an aliquot. Acidification with HCl destroyed the remaining borotritide and yielded a precipitate of dihydro-[5-^{3}H]FO. Following two washes with 1.0 mM HCl, the solid was redissolved in 25 mM ammonium acetate, pH 7.0, and bubbled with O_2. Reoxidation was effected either by addition of several milligrams of 5% Pt on asbestos or by several days exposure to ambient light. Lyophilization removed unbound tritium and buffer to give [5-^{3}H]FO with a specific activity of 17 mCi/mmol.

[5-^{3}H]FOH$_2$. Dihydro-[5-^{3}H]FO was made by reduction of tritiated FO. The reaction was effected with $NaBH_4$ in 0.1% Na_2CO_3 as described above. When the reduction was complete (as indicated by the loss of yellow color and blue-green fluorescence), the dihydro-FO was precipitated and washed in dilute HCl and then stored as an anaerobic aqueous slurry at 77 K. Alternatively, the reduction was performed with *Methanobacterium thermoautotrophicum* hydrogenase extract that had been passed through a DEAE column to remove anionic cofactors. In a typical preparation, 0.7 μmol of [5-^{3}H]FO (12 μCi) was dissolved in 0.5 mL of 0.1 M KP_i buffer, pH 7.5, containing 10 mM β-mercaptoethanol, degassed thoroughly under H_2, and treated with 0.5 mg of activated cell extract (Jacobson et al., 1982). After 30 min at 45 °C, the blue-green fluorescence of oxidized FO (under 313-nm illumination) had disappeared, and the reaction again was quenched by addition of 0.1 mmol of HCl. This denatured the protein and precipitated the reduced cofactor, which was washed and stored anaerobically.[2]

Enzymes

F420-reducing hydrogenase extract was isolated from *M. thermoautotrophicum* and resolved of anionic cofactors by passage through a DEAE column as described elsewhere (Jacobson et al., 1982).

M. thermoautotrophicum F420:NADPH oxidoreductase was isolated as a byproduct of the H_2ase purification by a modification of the method of Yamazaki & Tsai (1980). DEAE-Sephadex-resolved cell extract (Jacobson et al., 1982) was applied to a column of 2′,5′-ADP–Sepharose (Hiwatashi et al., 1977). The unbound hydrogenase was washed through with 50 mM Tris-HCl containing 50 mM KCl. Active oxidoreductase was eluted with 1 mM NADPH in the same buffer. Following a second DEAE column and a Sephadex G-100 step, approximately a 100-fold increase in specific activity was obtained with a 2% yield of a nonhomogeneous protein (Jacobson, 1981). As the purification was effected in the absence of reducing agents, it was necessary to activate the protein with 0.8 mM DTT (aerobically at room temperature for 3 h) prior to assay. These assays were done under argon (to suppress the high levels of NADPH and FOH_2 oxidase activities) in 50 mM KP_i, pH 6.0, containing 40 μM FO and 100 μM NADPH. At this pH, the final specific activity was 9.5 μmol min^{-1} mg^{-1} corresponding to 3.0 units/mg at pH 7.0 (Jacobson, 1981).

NADP(H):FMN oxidoreductase from *Benekea harveyii* was prepared and assayed as described in Fisher et al. (1976). Following storage at −20 °C for several years, the enzyme still retained 60% of the initial activity.

Flavodoxins from *Megaspherium elsdenii* and *Clostridium* strain MP were the gifts of Drs. V. Massey and M. Ludwig, respectively, and both were converted to their apoproteins by dialysis against KBr (Mayhew, 1971a,b).

Riboflavin kinase:FAD synthetase was isolated from *Brevibacterium ammoniagenes* by the procedure of Spencer et al. (1975).

Dihydroriboflavin kinase:FADH$_2$ synthetase from *Bacillus subtilis* (Kearney et al., 1980) was generously provided by Dr. E. Kearney.

Methods

FO/FOH$_2$ disproportion rate was measured by a modification of the method of Spencer et al. (1976). A 0.48-mL aliquot of buffer containing 48 nmol of [5-^{3}H]FO (770 nCi) was degassed under N_2. At t_0, 60 nmol of anaerobic $NaBH_4$-reduced FOH_2 (in 0.02 mL) was added, and reaction vials were placed, in the dark, in a water bath at 40 °C. At the indicated times, 0.05 mL was removed and immediately frozen in plastic tubes at 77 K. Reactions were carried out

[2] Enzymatically prepared FOH_2 was found to be more air labile than chemically reduced material. This is most probably a consequence of contaminating FAD (liberated during acid denaturation of the F420-reducing H_2ase) serving as an oxidation catalyst.

in either 0.1 M MES (pH 5.9), 0.1 M KP_i (pH 7.0), or 0.1 M KPP_i (pH 8.3).

The distribution of tritium was determined shortly thereafter by separating FO from FOH_2 on a Waters μBondapak C_{18} HPLC column with 25% MeOH/75% 5 mM ammonium acetate, pH 7.5, flowing at 2 mL/min. The respective bimolecular rate constants were derived from this data by the approach of Spencer et al. (1976) for 5-deazariboflavin.[3]

8-OH-5-dRF/FOH_2 reaction rate was measured on a Perkin-Elmer Model 557 UV–vis spectrophotometer in the dual-wavelength ($\lambda_1 - \lambda_2$) mode. At a given pH, appropriate wavelengths (i.e., 430 and 410 nm at pH 8.3) were chosen for FO (λ_{max} 420 nm) such that $Abs_1 - Abs_2 = 0$, effectively blinding the spectrophotometer to changes in [FO] as the reaction proceeds. Under these conditions, the 6-nm difference in the λ_{max} of 8-OH-5-dRF (426 nm) results in a new extinction coefficient e^* ($=e_{430} - e_{410}$) that is proportional to e_{426} and that was determined experimentally. This value was then used to measure the rates of 8-OH-5-dRF reduction, yielding k_2.

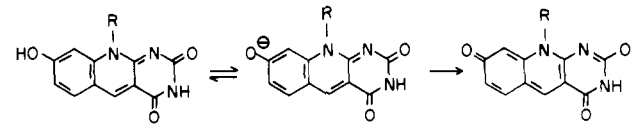
Sulfite titrations were carried out in either 100 mM MES, pH 6.4, 100 mM KP_i, pH 7.3, or 100 mM $NaPP_i$, pH 8.3, by using the method and analysis of Jorns & Hersch (1975). The concentration of SO_3^{2-}, calculated from the bisulfite pK_a of 6.9 (Muller & Massey, 1969) and the titration pH, was used to generate the K_{SO_3}. Absorbance changes were monitored at 386 nm for 8-MeO-5-dFl_{Et} and at 416 nm for 8-OH-5-dFl_{Et}.

FO redox potential was determined by H_2/H_2ase equilibration in double-septum sealed quartz cuvettes as described in Beinert et al. (1978). Incubations were carried out in 200 mM KPIPES, pH 7.0, with either 200 μM diquat or 20 μM FO. After thorough degassing under argon by cycles of repeated evacuation, manometrically determined mixtures of H_2 in Ar (ranging from 0.3 to 35%) were introduced into the cuvettes for the final filling. Approximately 50 μg of activated, anaerobic methanogen cell extract (DEAE resolved) was added to each, and the equilibrium was followed over the course of 20 h at room temperature. At that time, the concentrations of oxidized and reduced chromophores were determined from the absorbance changes (at 420 nm for FO and at 460 nm for DQ). The cell potential (E_{H_2}) at any partial pressure of H_2 (P_{H_2}) was calculated from the Nernst equation by using a slope of 30 mV for the two-electron donor.

Tritium Release in Reoxidation of [5-^{3}H]FOH_2. Volatilization of tritium from labeled FOH_2 was assayed in 0.5 mL of buffer ($NaPP_i$, pH 8.3). Oxidations were followed spectrophotometrically after addition of either 0.1 nmol of riboflavin or 0.1 mg of DEAE-resolved methanogen cell extract. Tritium washout was measured by lyophilizing the samples, individually, in a minature all-glass apparatus containing a 5-mL pear-shaped flask (with a 14/20 ground joint) connected to a vacuum trap immersed in liquid nitrogen. The solid residue was redissolved in 0.5 mL of water, and a portion (20%) was counted for 2 min in 3 mL of ACS scintillant (Amersham). The trap was emptied and washed with an additional 0.5 mL of water, and the entire volume was counted. Recovery of volatile tritium by this method was >95% against a ^3H_2O standard.

Stereochemical Analysis of B. harveyii Oxidoreductase. Flavin chirality of this enzyme was assayed by using the incorporation of tritium into ethanol with a coupled enzyme system. The oxidoreductase's demonstrated 4*R* selectivity toward NADH (Fisher & Walsh, 1976) and the need for a low isotope effect in the coupling enzyme led to the choice of horse liver alcohol dehydrogenase (HLADH). The reaction mixtures (0.57 mL) contained 0.2 mg of HLADH (Sigma), 1.2 μmol of acetaldehyde, and 0.2 μmol of NAD^+ in 100 mM $NaPP_i$, pH 8.65. Additionally, either 0.04 μmol of $NaBH_4$-reduced (racemic) or 0.06 μmol of H_2ase-reduced (chiral) [5-^{3}H]FOH_2 (16 nCi/nmol) was added, and the reaction vials were degassed under N_2 at 0 °C. To initiate, 45 μg of oxidoreductase was added anaerobically, and samples were incubated at 30 °C for 30 min prior to lyophilization analysis as described above. With this level of protein, the FOH_2 oxidation reaction proceeds to 90% completion. With the racemate, only 13% of the label was released as ethanol while the chiral sample (containing ^{3}H only on the "B" side) transfered 30–40%, thereby establishing its B side specificity.

Scheme I

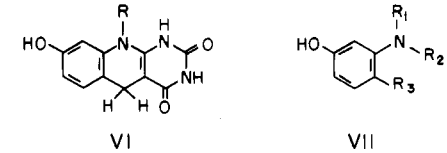


V

Results

Spectroscopic and Structural Properties. Acid hydrolysis of factor 420 produces the riboflavin level, FO. The chromophore's initial structure assignment (Eirich et al., 1978) was confirmed by comparison of this acid hydrolysis product with synthetic 7-demethyl-8-hydroxy-5-deazariboflavin (Ashton et al., 1979). FO prepared by that synthesis has been used in the experiments described in this paper.

Eirich et al. (1978) noted that the 420-nm peak in F420 represents a 50-nm bathochromic shift (from 472 nm) in the absorbance maximum of 8-OH-RF characteristic of the 5-aza to 5-carba (O'Brien et al., 1970; Edmondson et al., 1972) substitution. As with 8-OH-RF (Ghisla & Mayhew, 1973, 1976), facile deprotonation of the hydroxyl leads to the conjugated paraquinoid anion (λ_{max} = 420 nm), V (Scheme I), and we obtained similar values for this pK_a (5.85 vs. 6.05), for the N(3) pK_a (11.6 vs. 11.3), and for the anion's extinction coefficient (37 500 vs. 39 000 M^{-1} cm^{-1}) as those published for FO (Eirich et al., 1978). In that paper, UV and NMR spectra were used to show that, like 5-dRF, reduction of FO produces a 1,5-dihydro-5-deazaisoalloxazine (VI). N(1) is therefore



VI VII

slightly acidic, with a pK_a for FOH_2 of about 7 [7.2 (Eirich et al., 1978); 6.9 (this work)]. Reduction of FO interupts conjugation and isolates the benzenoid portion of the molecule, converting it to an alkyl-substituted *m*-aminophenol, VII. The hydroxyl pK_a of FOH_2 was measured by titration to be 9.7 (Jacobson, 1982), a value comparable to the 9.9 pK_a for *m*-aminophenol (Jencks & Regenstein, 1976).

The dependence of the absorption spectrum on solvent polarity has been studied with flavins, 1-deazaflavins, and the zwitterionic 5-alkylflavin semiquinone (Harbury et al., 1959; Muller et al., 1972; Spencer et al., 1977). With the neutral species 1-dRF, decreasing solvent polarity shifts the transitions

[3] Under initial conditions, only hydride is available for transfer from FOH_2; however, once formation of [5-^{3}H]FOH_2 has occurred, tritium can be transfered back to FO. Although this reaction will contribute to racemization of the sample, it is kinetically silent since products and reactants are identical.

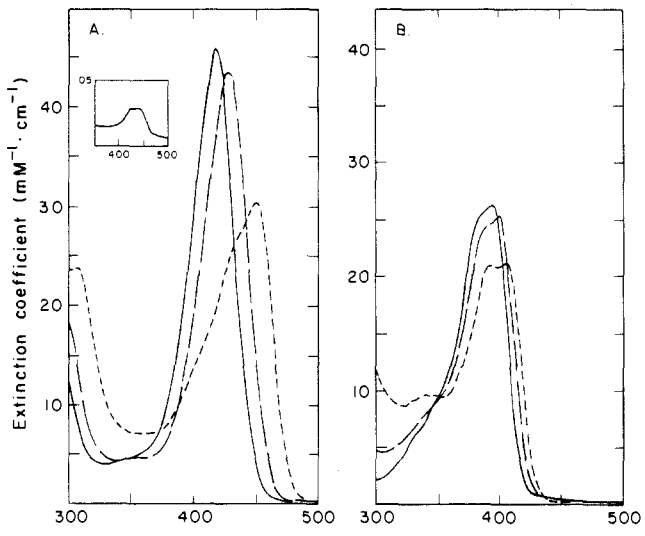


FIGURE 1: UV–visible spectrum dependence on solvent polarity. FO spectra were recorded in solutions containing either 0% (solid), 50% (long dashes), or 100% Me_2SO (short dashes). (A) All solutions contained 0.1% Et_3N as a base. (Inset) Spectrum of *S. griseus* photoreactivation enzyme (Eker, 1980). (B) All solutions contained 0.1% acetic acid to keep FO fully protonated.

to higher energy (shorter wavelength) with increased resolution of the component bands. Panel B of Figure 1 demonstrates that the spectrum of the protonated, neutral FO exhibits little wavelength shift or increased resolution, in 100% Me_2SO (dashed line). In the same solvents, its conjugate base, IV (Figure 1A), exhibits a pronounced shift in absorbance maximum to lower energy (from 420 to 450 nm), which is predicted for a charged species in a hydrophobic environment. As suggested by the inset in this figure, the F420-like cofactor bound to the *S. griseus* photoreactivation enzyme (Eker, 1980) possesses a visible spectrum similar in both λ_{max} and peak shape to that of the FO anion in 100% Me_2SO. This stabilization of the cofactor's anion in an apolar milieu pushes its absorbance further into the visible range, consistent with its postulated role in the photoreversion of thymine dimers in DNA.

Sulfite Adduct Formation. The effective electrophilicity of N(5) in free or protein-bound flavins and flavin analogues, as measured by formation of the reversible sulfite adduct, has been shown to correlate well with the measured redox potential (Massey et al., 1969; Jorns & Hersh, 1975). Hemmerich et al. (1977) has pointed out that both the nicotinamides and the 5-deazaflavins form similar adducts [at C(4) and C(5), respectively]. When they plotted redox potentials and K_d for a variety of flavin and nicotinamide analogues, they found that (Figure 2B) one line contained the flavin data while a second line apparently contained both the nicotinamide and 5-deazaflavin points.

From the model compounds 10-ethyl-8-hydroxy-5-deazaflavin and 10-ethyl-8-methoxy-5-deazaflavin, sulfite K_ds were determined by spectroscopic titration at three pH's (Figure 2A). These results demonstrate that blockage of the ionizable hydroxyl affects adduct formation, with the difference in the slope (open stars vs. open circles) reflecting the varying proportion of 8-OH-5-dFl_{Et} present as the protonated, electrophilic form. The K_ds are equal at the intersection of the two lines, and this is extrapolated to occur at a pH (5.7) slightly below that of the pK_a (5.9), where the fraction of the nonreactive anion becomes small. If the K_ds of 8-OH-5-dFl are calculated from the concentration of the protonated fraction, rather than the total deazaflavin concentration (solid stars), a line is derived that coincides with that of the 8-methoxy compound.

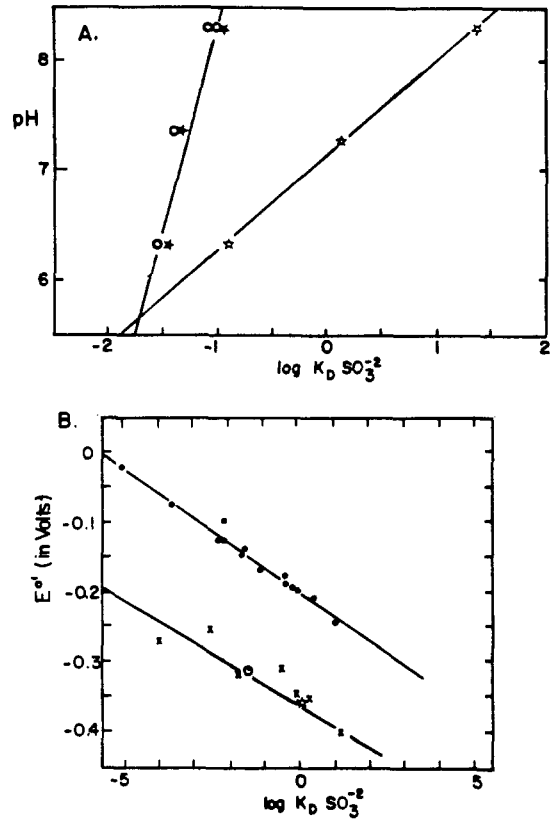


FIGURE 2: Sulfite K_d for 8-hydroxy- and 8-methoxy-5-deazaflavins. (A) Dissociation constants were determined for both the *N*(10)-ethyl-8-hydroxy-5-deazaisoalloxazine (open stars) and the 8-methoxy derivative (open circles) at the indicated pHs. When the K_d of the 8-OH compound was adjusted to reflect the mole fraction of the protonated species alone (solid stars), a second line was obtained, coincident with that of the 8-methoxy derivative. (B) Figure 1 from Hemmerich et al. (1977) is reproduced here with the addition of the K_ds determined above at pH 7.3.

The linear free-energy relationship from Hemmerich et al. (1977) is reproduced in Figure 2B with addition of the pH 7.3 data from above. The midpoint potential of FO, determined below (−350 mV), was used for 8-OH-5-dFl while that of 5-dRF [−310 mV (Fisher et al., 1975)] was used to estimate the E_0' of 8-MeO-5-dFl. With these numbers, an excellent agreement was obtained between the measured K_ds and the nicotinamide/5-dFl line from Hemmerich et al. (1977).

Redox Properties. Although Eirich et al. (1978) concluded that both chemical ($NaBH_4$) and enzymatic (H_2ase) reductions result in a reduced species with a 1,5-dihydro-5-deazaflavin structure, it was important for later stereochemical experiments to confirm that labeled $NaBH_4$ would introduce isotope specifically at C(5). Following reduction of FO (with either $NaBH_4$ or $NaBD_4$) and reoxidation (on Pt/asbestos or with catalytic riboflavin), NMR analysis indicated that the H(5) peak was selectively diminished in intensity by almost 70% when BD_4^- was used. Two further reduction/oxidation cycles with BD_4 resulted in virtual disappearance of the H(5) signal,[4] in accord with the results we have previously reported for 5-dRF (Spencer et al., 1975).

Like 5-$dRFH_2$, chemically reduced FO (via $NaBH_4$ or $Na_2S_2O_4$) is only slowly reoxidized by molecular oxygen. At

[4] Prolonged incubation of FO in basic D_2O results in collapse of the H(6) doublet to a singlet and loss of the H(7) signal, apparently caused by exchange of the proton on H(7) with solvent deuterium. This is likely explained by some reprotonation of the anionic paraquinoid at C(7) rather than O(8), leading to the observed label incorporation.

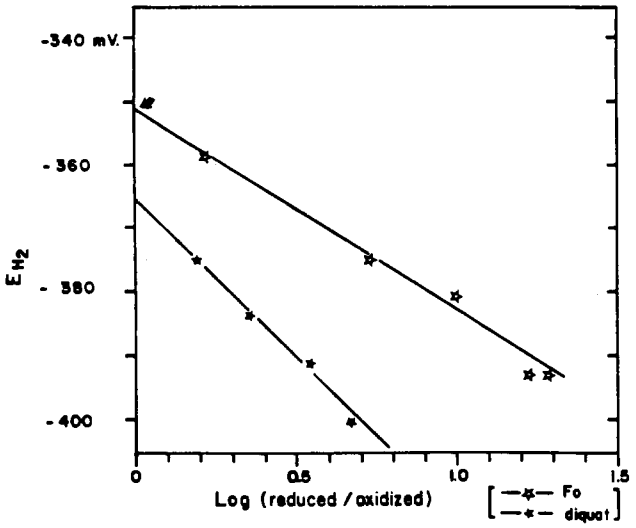


FIGURE 3: FO redox potential by H_2/H_2ase equilibrium. FO (open stars) and diquat (solid stars) were reduced under mixtures of H_2 and argon with methanogen hydrogenase extract as described under Methods.

pH 7, in the dark, the apparent half-time for reoxidation in air-saturated solution is about 22 h at room temperature. Again like 5-dRFH_2, the autooxidation rate is also slower at higher pH ($t_{1/2}$ of 60 h, pH 9.6) and is accelerated 7–10-fold by ambient light. FOH_2 and 5-dRFH_2 both react slowly with the obligate one-electron acceptor cytochrome *c* (for FOH_2, k = 1.2 M^{-1} s^{-1} vs. 20 M^{-1} s^{-1} for 5-dRfH_2). The thermodynamic accessibility of both one- and two-electron pathways permits rapid two-electron reactions to occur between dihydro-5-deazaflavins and either phenazine methosulfate (k for FOH_2, 800 M^{-1} s^{-1}) or riboflavin (1200 M^{-1} s^{-1} with FOH_2). The last reaction is described in detail below.

Redox Potential. Two prior, but somewhat contradictory, reports on the cofactor's reduction potential have been cited without experimental detail. Under their Discussion, Eirich et al. (1978) cited a value of −370 mV while a later study by Pol et al. (1981) yielded values of −340 mV for F420 and −350 mV for the analogue 8-hydroxy-10-methyl-5-deazaisoalloxazine. In collaboration with Drs. W. Orme-Johnson and L. Daniels, we have now used the methanogen F420-linked hydrogenase (Jacobson et al., 1982) to catalyze FO equilibration with known partial pressures of H_2. Bacterial hydrogenases (e.g., those from *M. elsdenii* and *Clostridium pasteurianum*) have previously been used in similar potentiometric studies of dithionite and various ferridoxins (Mayhew, 1973; Stombaugh et al., 1976).

The results of one such experiment are shown in Figure 3. As anticipated for the two-electron acceptor FO (open stars), the Nernst plot exhibits a slope close to −30 mV per decade. By contrast, reduction of the single-electron acceptor diquat (DQ, solid stars) has a slope of −54 mV per decade. The equilibrium potentials, derived from the *y* intercepts, are −353 for FO and −365 mV for diquat. The former value agrees well with the work of Pol (1981), and the DQ potential similarly agrees with the literature (Summers, 1980).

By use of the *M. thermoautotrophicum* F420:NADP oxidoreductase (Jacobson, 1982) as catalyst, FO was also equilibrated with known mixtures of NADP and NADPH (E_0' = −320 mV). This method provided a redox potential of −340 mV (data not shown).

Oxidation of FOH_2 by Riboflavin Analogues. The rate of dihydro-5-deazariboflavin oxidation by a variety of flavin analogues has been shown to vary exponentially with the acceptor's redox potential (Spencer, 1978; Blankenhorn, 1976). Similarly, the rate of reaction between flavins and dihydronicotinamides (Blankenhorn, 1976) is directly related to the free-energy difference between the reactants. As demonstrated in Figure 4, oxidation of FOH_2 demonstrates similar behavior over a wide range of potentials.

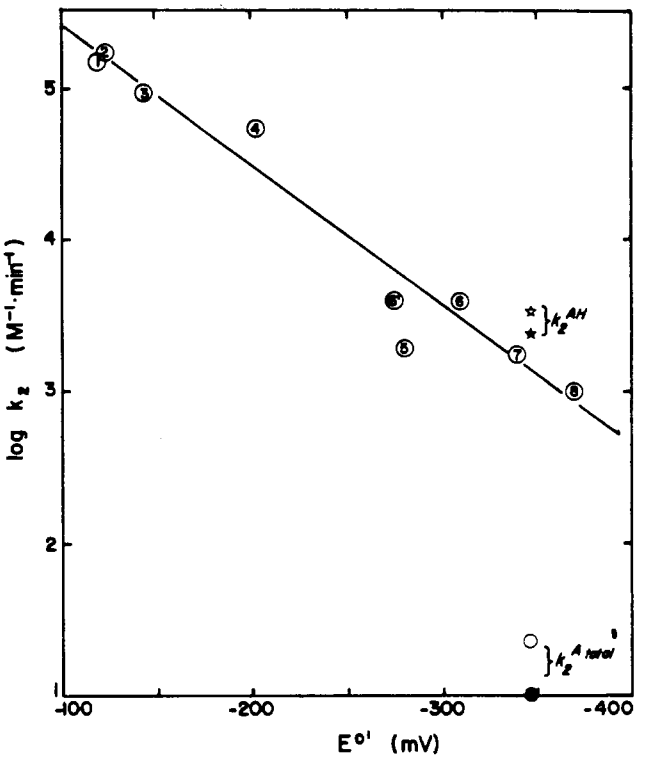
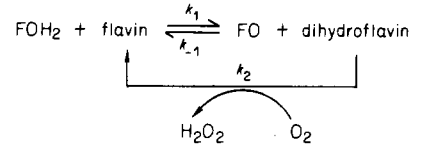


FIGURE 4: FOH_2 oxidation by various riboflavin analogues. The rates of dihydro-FO oxidation by various flavin analogues are plotted as a function of their redox potentials. For the numbered analogues, the redox potentials and rate measurements were as described in Walsh et al. (1978). The rates for FO (solid symbols) and 8-OH-5-dRF (open symbols) were determined as described under Methods, and the FO redox potential (this work) has been used for both compounds. For these two compounds, the circles represent k_2s based on the total concentration of the acceptor flavin. The values represented by the stars have been calculated to account for the mole fraction of protonated acceptor (0.35%) present at the assay pH (8.3). The numbered analogues are (1) 2-SH-RF, (2) 7-Cl-RF, (3) 8-Cl-RF, (4) RF, (5) 1-dRF, (6) 5-dRF, (7) 8-OH-RF, and (8) 5-dRFH_2.

Scheme II



Any reduced flavin that is then rapidly autooxidizable will catalytically reoxidize FOH_2 (in Scheme II, $k_2 \gg k_1$). Those tested here include RF, 1-dRF, 8-OH-RF, 2-thio-RF, and both 7-Cl- and 8-Cl-RF. Oxidized 5-deazariboflavins will also react; however, the slow subsequent autooxidation of their dihydro forms ($k_2 \ll k_1$) makes these reactions stoichiometric, rather than catalytic. Regardless, the reaction rates with 5-dRF and 5-dRFH_2 agree well with those obtained with the 5-azaflavins, suggesting a common redox mechanism such as hydride transfer (Blankenhorn, 1976).

The disproportionation between reduced and oxidized FO forms a special case of the generalized flavin reaction considered above (where $k_1 = k_{-1}$), which will be discussed in detail below. When this rate constant is calculated from the concentration of protonated FO (k_2^{AH}) present at the reaction

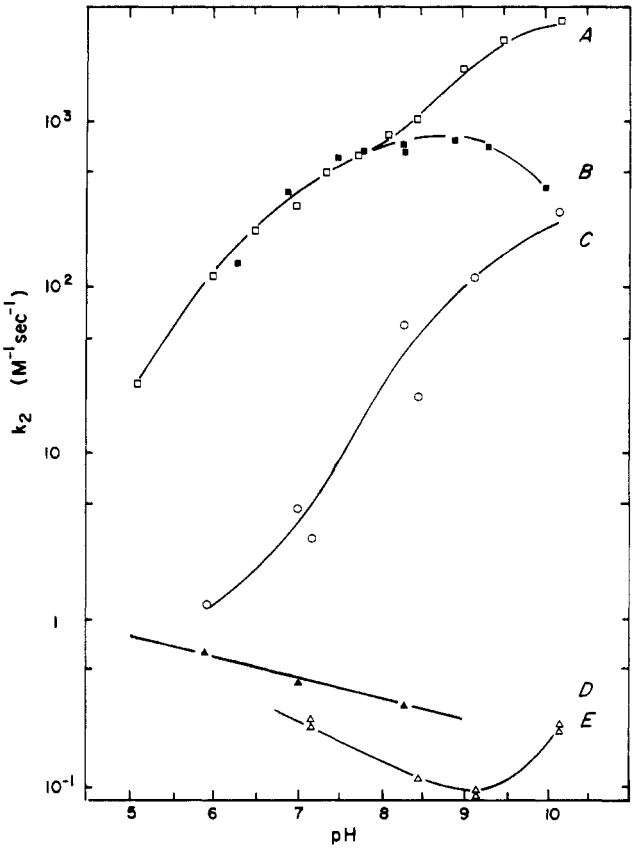
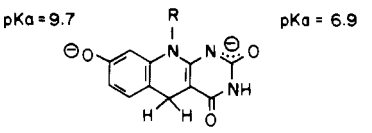


FIGURE 5: pH dependences of dihydro-5-deazaflavin reoxidations. The rates of flavin- and deazaflavin-catalyzed oxidation of FOH_2 and 5-dRFH_2 are plotted as a function of pH. In curves A and B, riboflavin-catalyzed oxidations of FOH_2 (open squares) and 5-dRFH_2 (solid squares) were performed in a mixed buffer containing 25 mM each of KP_i, $NaPP_i$, sodium acetate, and glycine. Reactions contained 10–40 μM dihydro-5-deazaflavin and 1–5 μM riboflavin. Curves D and E represent the reaction of FOH_2 with FO (solid triangles) and with 8-OH-5-dRF (open triangles) as described in the text. Curve C represents a transformation of the data in curves D and E that assumes that only the protonated FO or 8-OH-5-dRF can serve as acceptor.

pH (0.35% at pH 8.3) rather than total FO (k_2^{A+AH}), a significantly improved fit to the flavin line is obtained as noted in Figure 4. As with sulfite adduct formation, this again suggests that only the protonated cofactor is reactive toward electrophilic attack, in this case by a hydride equivalent at C(5).

The anticipated importance of FO interactions with flavoproteins [e.g., the reduction of FO or F420 by the FAD-containing methanogen hydrogenase (Jacobson et al., 1982)] led us to investigate the pH dependence of riboflavin/FOH_2 oxidoreduction. The pH/rate behavior for the synthetic analogue dihydro-5-dRF had previously been studied in this laboratory by Fisher (1977). This experiment was repeated to permit direct comparison with FOH_2 reactivity, and these results are shown in Figure 5. The 5-dRFH_2 rate profile, below pH 8.5, has been postulated to derive from the reactivity of only the N(1) anion as redox donor (Blankenhorn, 1976; Spencer et al., 1975)—the same is apparently true of FOH_2. Previous workers suggested that N(3) deprotonation of the acceptor riboflavin causes the rate decrease observed above pH 9.5 (Suelter & Metzler, 1960; Spencer et al., 1975). In this pH region, the dramatic increase in FOH_2 reaction rate requires that the FOH_2 dianion, VIII (pK_a = 9.7), have substantially higher hydride donor activity than the conjugate monoanion (pK_a = 7.0)—enough to overcome the deactivating ionization of RF at N(3). This point will be further elaborated



VIII

below in a discussion of the self-exchange reaction.

Chiral Stability of Dihydro-8-hydroxy-5-deazaflavins. By analogy to C(4) of NADH, the C(5) position of the central dihydropyridine ring in 5-dRFH_2 is prochiral. We have noted that the rapid disproportionation between reduced and oxidized 5-deazaflavin (k_{11} = 22 M^{-1} s^{-1} at 0 °C) (Fisher et al., 1975) leads to rapid racemization, and this fact previously had prevented use of 5-^{2}H-labeled or 5-^{3}H-labeled dihydro-5-deazaflavins as chiral probes of flavin redox reactions. In order to determine if chiral processing of labeled FO species could be observed, we have measured the reaction rate between FOH_2 and [5-^{3}H]FO (by HPLC) and between FOH_2 and 8-OH-5-dRF (spectroscopically) over a range of pH's. These results have been incorporated into Figures 4 and 5. As shown there, the two methods yield comparable rates, with FO being about 2-fold faster than its 7-methyl analogue (8-OH-5dRF) in oxidizing FOH_2.

In Figure 4, a poor fit to the free-energy correlation line is obtained when the total concentration of oxidized FO (or 8-OH-5-dRF) is used to calculate the rate constant (k_{11} = 600 and 1200 M^{-1} s^{-1} for FO and 8-OH-5-dRF, respectively). The improved fit that results from using the concentration of protonated 8-OH-5-deazaflavin again suggests that only the neutral oxidized species functions as hydride acceptor in this reaction. Thus at neutral pH, the presence of the 8-hydroxy substituent retards self-disproportionation by 60–120-fold as compared with the parent 5-dRF. The consequent enantiomeric stability of [5-^{3}H]FOH_2 has permitted the enzymatic reduction and isolation of chiral FOH_2 for use in stereochemical experiments described below and in our previous paper on the methanogen F420:NADP oxidoreductase (Yamazaki et al., 1980).

As the reaction pH is varied from 6 to 9 and the mole fraction of protonated acceptor diminishes, the apparent rate constants (based on total acceptor concentration) decreases with a slope of approximately −0.2. By contrast, the rates of reaction with RF (Figure 5, curve A) and 5-dRF (data not shown) increase with increasing pH. The breakpoint seen above pH 9 in the 8-OH-5-dRF data (curve E) is again suggestive of increased reactivity for the dihydro-FO dianion (pK_a = 9.7). If both sets of rate constants are transformed to compensate for the changing mole fraction of protonated acceptor, a new curve is obtained with an increasing slope similar to that of FOH_2 reaction with RF (Figure 5, curve C).

Stereochemical Analysis of Enzymatic 5-Deazaflavin Reactions. The stereochemical stability of [5-^{3}H]- and [5-^{2}H]FOH_2 noted above has permitted the synthesis and isolation of chiral dihydro-[5-^{3}H]FO. Following reduction of [5-^{3}H]FO under H_2, by the *M. thermoautotrophicum* hydrogenase, the reaction was acidified at 0 °C to yield a beige precipitate that was washed and stored frozen as an anaerobic aqueous slurry. The dihydro-5-deazaflavin so obtained should contain protium only in the position activated by the enzyme. This is arbitrarily designated as the A side, by analogy to the convention used for relative chirality with nicotinamides prior to the establishment of absolute stereochemistry.

That methanogen hydrogenase was behaving as a chiral reductant of FO was confirmed as follows. Both H_2ase-generated [5-^{3}H]FOH_2 and racemic [5-^{3}H]FOH_2 (made by

Table I: Labilization of Tritium from [5-^{3}H]FOH_2

	volatile tritium		nonvolatile tritium		total tritium
	nCi	%	nCi	%	(nCi)
(A) Volatilizations of Tritium from $NaBH_4$-Reduced [5-^{3}H]FO[a] oxidation catalyst					
control (no addition)	0.3	1	30.5	99	31
H_2ase extract	15.2	51	14.5	49	30
riboflavin	2.2	8	26.5	92	29
(B) [5-^{3}H]FOH_2 Produced by $NaBH_4$ or by H_2ase, Air Oxidized in the Presence of Crude H_2ase Extract					
FOH_2 source					
borohydride reduced	4.0	47	4.6	55	8.4
after 1-month freezing	8.8	49	9.0	50	17.8
H_2ase reduced	0.3	4	6.7	97	6.9
after 1-month freezing	1.5	9	15.8	96	16.5

[a] Performed as described under Methods.

Table II: Flavin Chirality of Flavin-Utilizing Enzymes

enzyme	source	relative chirality
F420-reducing H_2ase	*M. thermoautotrophicum*	A
F420:NADPH oxidoreductase	*M. thermoautotrophicum*	A
F420:NADPH oxidoreductase	*M. vannielli*	A
FMN:NADPH oxidoreductase	*B. harveyii*	B

$NaBH_4$ reduction of [5-^{3}H]FO) were air oxidized by a highly active dihydro-5-deazaflavin oxidase observed in aerobically prepared methanogen cell extract (Jacobson, 1982). This heat-labile activity removed, as ^3H_2O, 50% of the tritium initially bound to the racemate, while only control levels were released from the chiral sample (Table IB).

This result affords two conclusions; the first is that both the oxidase and hydogenase interact with the same face of the cofactor. The second conclusion, that the oxidase exhibits no product isotope effect during turnover, provides a convenient assay for stereochemical purity since all ^3H on the A side of [5-^{3}H]FOH_2 is converted to ^3H_2O. By contrast, the nonenzymatic riboflavin-catalyzed oxidation of racemic [5-^{3}H]FOH_2 labilized only 12% of the bound tritium during essentially quantitative reoxidation, indicating that the primary kinetic isotope effect in this chemical reoxidation conditions tritium distribution in the products (Table IA). This renders the riboflavin-catalyzed oxidation of the [5-^{3}H]FOH_2 species useless as a probe for ^3H distribution at the prochiral C(5) locus.

Using these two methanogen-derived enzymic activities to define the A side of [5-^{3}H]FOH_2, we have determined the relative stereochemistries of three other enzymes. As seen in Table II, the two F420:$NADP^+$ oxidoreductases from *M. vannielli* (Yamazaki & Tsai, 1980; Yamazaki et al., 1980) and *M. thermoautotrophicum* (Jacobson, 1981) share this specificity, whereas the NAD(P)H:FMN oxidoreductase from *B. harveyii* (Duane & Hastings, 1975; Fisher & Walsh, 1974) catalyzes hydride transfer to and from the opposite face of FO and so is definable as a B side enzyme. A combination of the methanogen and the *B. harveyii* enzymes will thus allow stereochemical assay for any redox reaction of FO or the natural cofactor, F420. The absolute chirality at C(5) of FOH_2 remains unassigned.

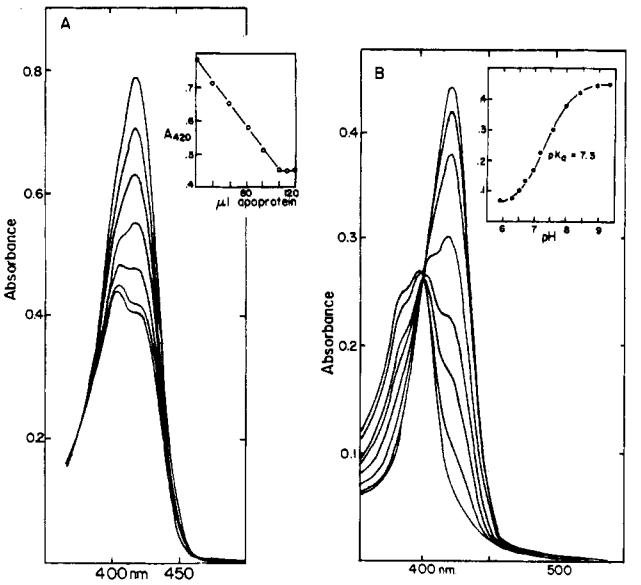


FIGURE 6: (A) Additions of clostridial apoflavodoxin (170 μM) were made to 1 mL of a 20 μM F^+ stock solution in 0.1 M KP_i, pH 7.0. (Inset) A_{420}, corrected for dilution, is plotted against the amount of added enzyme. (B) Following titration of F^+ with 205 μM *M. elsdenii* apoflavodoxin and removal of nonbound F^+ by passage through a small Sephadex G-25 column (in 50 mM KP_i, pH 7.0), concentrated $NaPP_i$, glycine, and HCl were added bringing the buffer to 50 mM in all three species and the pH to 4.2. Additions of 5 M NaOH were made, and the spectrum and pH were recorded. (Inset) Dilution-corrected A_{425} plotted as a function of the pH.

Interaction of 8-Hydroxy-5-deazaflavins with Non-Methanogen Proteins. The broad specificity of the *B. ammoniagenes* riboflavin kinase:FAD synthetase toward modified riboflavins has made it the method of choice for preparation of riboflavin analogues at the FAD level (Spencer et al., 1976; Walsh et al., 1978). Although 8-OH-5dRF is slowly converted to its 5'-ADP derivative (8-OH-5-dFAD) by this enzyme complex, its 7-demethyl form (FO) is not a substrate at either acidic or basic pH (Ashton et al., 1979). During 2-day incubations, neither its FMN form (F^+) nor its FAD form accumulates. The FAD level of FO also was not produced when chemically synthesized F^+ was used as substrate. The 7-methyl group is apparently important for substrate recognition.

Next, the dihydroriboflavin kinase:synthetase from *Bacillus subtilis* (Kearney et al., 1979) was tested with both FOH_2 and F^+H_2 as substrates. After incubation and then catalytic reoxidation with riboflavin, HPLC analysis detected only the FMN species, F^+. This indicates that only the dihydroflavin kinase component recognizes the reduced 7-demethyl-8-hydroxy-5-deazaisoalloxazine ring. Similar specificity had previously been reported when 5-$dRFH_2$ was tested by Kearney et al. (1979). To date, the FAD level of FO has not been reported in either methanogens or streptomycetes.

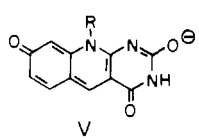
To determine whether flavin-binding proteins would recognize the 7-demethyl-8-hydroxy form of 5-deazaflavins, the binidng of FO to the apo form of the egg white riboflavin binding protein (Beckvar, 1973) and of F^+ to the apoflavodoxins of *Clostridium* strain MP and *M. elsdenii* (Mayhew, 1971a,b; Mayhew & Massey, 1969) has been assayed spectrophotometrically at neutral pH. In each case, addition of apo protein led to fluorescence quenching and to absorbance changes reminiscent of those obtained upon protonation of the 8-hydroxyl (Figure 6A). This apparent stabilization of the neutral cofactor was confirmed by pH titration of the *M. elsdenii* flavodoxin complex. The pK_a shift to 7.3 (Figure 6B) is of the same magnitude (1.3 pH units) as that reported for 6-OH-FMN (Mayhew et al., 1974) and for 8-OH-FMN

(Ghisla et al., 1976). In addition, each protein confers a small (5-nm) red shift on the absorbance maximum of the anionic form of the bound cofactor.

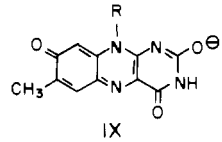
Discussion

5-Carba-5-deazariboflavin was originally prepared as a synthetic structural analogue of the natural parent riboflavin in the hope that 5-carba for 5-aza substitution would provide insight into flavoprotein catalysis (O'Brien et al., 1970; Edmondson et al., 1972; Fisher et al., 1976). Though its electronic spectrum closely approximates that of riboflavin, with the characteristic twin absorbance peaks each shifted 50 nm toward the blue, the 5-dRF molecule is chemically quite distinct. The structural switch from a central pyrazine to pyridine ring confers an inability to undergo facile one-electron reactions in the ground state. This fact, the attendant low redox potentials, and kinetic sluggishness of dihydro forms in reactions with O_2 have been interpreted (Massey & Hemmerich, 1980; Spencer et al., 1976) to show that the 5-deazaflavin skeleton is chemically more nearly analogous to nicotinamide- than to riboflavin-based coenzymes.

8-Hydroxyriboflavin, by contrast, was first isolated at the 8-OH-FAD level as a tightly bound orange component during purification of the electron-transfering protein from *M. elsdenii* (Ghisla & Mayhew, 1973). From studies with synthetic model compounds, Ghisla and co-workers concluded that above pH 6, the single long-wavelength absorbance (at 476 nm) could be attributed to the delocalized paraquinoid anion, IX. Al-



V



IX

though neither protonated nor anionic 8-OH-RF resembles riboflavin in electronic spectrum, they share with it a number of important chemical features, such as rapid autoxidation of their dihydro forms and facile one-electron redox reactivity.

In their initial report on the methanogen coenzyme F420, Cheeseman et al. (1972) described the rapid autooxidation of crude extract reduced cofactor. This was somewhat paradoxical in light of its subsequent structure elucidation as an 8-hydroxy-5-deazariboflavin (Eirich et al., 1978), since we have previously noted that 5-dRFH_2 is only slowly oxidized in air (Spencer et al., 1976). This, among other facts, stimulated our research effort to understand the relative contributions of the 5-carba and the 8-hydroxy substitutions to the properties of the methanogen cofactor.

Both the protonated and O(8)-methylated forms of the F420 chromophore (λ_{max} = 400 nm) are strikingly similar to 5-dRF in C(5) electrophilicity toward sulfite and in their rates of chemical reduction by borohydride and dithionite. As in 8-OH-RF, deprotonation above pH 6 produces the electron-rich paraquinoid anion (V), which appears to lack electrophilicity. As demonstrated above, the pH dependence of SO_3^{2-} addition and of the disproportionation/self-exchange reaction (between FO and FOH_2) can best be modeled by assuming that the FO anion is nonreactive. A major practical consequence of the low reactivity of the oxidized FO anion (99% of the total FO at pH 8) is that solutions of FOH_2, in the presence of FO (anion), are much slower to disproportionate (50–100-fold) than in the complimentary 5-dRFH_2/5-dRF system. This means that chiral [5-^{3}H]FOH_2 species have a long enough half-life to be generated enzymatically and then be analyzed for stereochemistry. Such a line of investigation is not open to the 5-dRF system, which scrambles much more rapidly. This is a key modulation, which the 8-hydroxy substituent imparts to the FO and F420 structures.

Reduction of FO produces the anticipated 1,5-dihydro-5-deazaisoalloxazine structure, interrupting the extended conjugation of the oxidized flavin. This isolates the 8-hydroxyl, raising its pK_a from that of a strongly electron-deficient phenol (5.8) to that of a normal phenol (9.7). Below pH 9 [where the O(8),N(1) dianion is not important], this makes the properties of FOH_2 virtually identical with those of 5-dRFH_2. In the reduced form, at physiological pH, the 8-hydroxy substituent has a much smaller effect on reactivity than in the oxidized form. Both dihydro-5-deazaflavins are slow to oxidize aerobically in the dark ($t_{1/2}$ ca. 20–40 h), and these reactions are both stimulated by ambient light (Edmondson et al., 1972). Additionally, their rates of riboflavin-catalyzed reoxidation are similar below pH 8.5 (Figure 5), and both exhibit similar linear free-energy changes as expressed in rate changes when the redox potential of the acceptor flavin is varied. In aggregate, such results lead us to conclude that there is no major difference between the reactivities of the dihydro forms of FO and synthetic 5-deazariboflavin: the central dihydronicotinamide ring controls the chemistry of the tricyclic system (Hemmerich & Massey, 1980; Spencer et al., 1976).

The interaction of flavin coenzymes with FO or F420 has an important physiological consequence for the methanogens. One key function of flavin coenzymes is to interface two-electron donor/acceptors (such as nicotinamides) with one-electron redox reagents like O_2, heme, or Fe/S clusters. All hydrogenases so far purified contain iron/sulfur centers (Adams et al., 1981), and the enzyme from *Alcaligenes eutrophus*, which can directly reduce NAD, also contains bound FMN (Schneider & Schlegel, 1978). Recently, we have purified the F420-reducing hydrogenase from *M. thermoautotrophicum* and shown it to contain stoichiometric amounts of bound FAD as well as Fe/S centers and Ni(III) (Jacobson et al., 1982; Kojima et al., 1983). The reported air lability of cell-extract-reduced F420 (Cheeseman et al., 1972) can be explained not by an intrinsic susceptibility of F420H_2 to autoxidation but by the presence of a potent dihydro-5-deazaflavin oxidase activity associated with the hydrogenase (Jacobson et al., 1982). On purification of the FAD-containing hydrogenase 25-fold (to ca. 50% homogeneity), the F420H_2 oxidase activity copurifies coordinately (Jacobson, 1981). This oxidase activity, along with the data on riboflavin-catalyzed oxidation of FOH_2, suggests proximity of the F420 binding site to the FAD site in the hydrogenase.

Our determination of the midpoint potential for the FO/FOH_2 redox couple were performed by equilibration with two methanogen enzymes, the F420-reducing hydrogenase and the NADP:F420 oxidoreductase from *M. thermoautotrophicum*, and gave values of −340 and −350 mV. These are consistent with values recently obtained by Pol et al. (1981) for the 7-demethyl-8-hydroxy-5-deazalumiflavin (−350 mV) and F420 itself (−340 mV). An independent estimate of the E_0' for F420/F420H_2 can be obtained by using kinetic constants from studies on the *M. vanielli* F420-NADP oxidoreductase at pH 7.0 (Yamazaki & Tsai, 1980) and the appropriate Haldane relationship. Isotope-transfer experiments with this enzyme (Yamazaki et al., 1980) have suggested a sequential mechanism, which determines the form of the Haldane equation to be

$$K_{eq} = V_{max(for)}K_{m(P)}K_{m(Q)}/[V_{max(rev)}K_{m(A)}K_{m(B)}]$$

for the reaction

$$A + B \underset{rev}{\overset{for}{\rightleftharpoons}} Q + P$$

Substitution of the four K_m and two k_{cat} values allows calculation of K_{eq} for the F420/NADPH system. This yields an E_0' of −351 mV for F420.

The 8-hydroxy and 5-carba substitutions lower the redox potential of the F420/F420H$_2$ couple some 140–150 mV relative to the corresponding flavin/dihydroflavin couple and place it in a different redox niche. Whereas flavins are reduced by nicotinamides ($K_{eq} = 10^3$), the 8-hydroxy-5-deazaflavin cofactors serve as redox shuttles to be reduced by H_2 (via hydrogenase action; $K_{eq} = 10^3$) and in turn to reduce NADP ($K_{eq} = 10$). Whether the 5-carba or the 8-hydroxy substitution is more important is difficult to factor out since E_0' for the 8-hydroxyriboflavin couple is −340 mV (Ghisla & Mayhew, 1979) while that for 5-dRF has been reported as −280 (Stankovich & Massey, 1976) or −310 mV (Fisher et al., 1975). However, it is probably the 5-carba substitution with its attendant nicotinamide-type chemistry that requires that the methanogen hydrogenase contain bound FAD to pass electrons to F420 and FO, just as the *A. eutrophus* H_2ase needs bound FMN to reduce NAD.

In a preliminary paper on direct transfer of tritium between [4-^{3}H]NADPH and FO or F420 by the *M. vanielli* oxidoreductase (Yamazaki et al., 1980), we have noted the similarity between C(4) of nicotinamide and C(5) of FO as a site for hydride transfer. In particular, both C(4) of NADPH and C(5) of FOH$_2$ and F420H$_2$ are diastereotopic, prochiral methylene centers. The determination of relative and then absolute stereochemistry in nicotinamide coenzyme utilizing dehydrogenases was an important probe of mechanism and continues to be a subject of investigation (Benner, 1982) for understanding catalysis. In this paper, we have noted that the [5-^{3}H]FOH$_2$ species have a sufficient lifetime in aqueous solution, in the presence of oxidized FO, that relative stereochemistry (A side vs. B side) can be determined. We have observed A side specificity for methanogen hydrogenase and two NADP:F420 oxidoreductases and the opposite B side specificity for the *B. harveyii* NAD:FMN oxidoreductase (the feeder enzyme for bacterial luciferase).

The chiral integrities of [5-^{3}H]- or [5-^{2}H]FOH$_2$ species are kinetically constrained by a chirality-scrambling disproportionation when oxidized FO molecules are present, since this redox process goes by hydride transfer between FO and FOH$_2$. By contrast, this is not a significant problem for [4-^{3}H]NADH or [4-^{3}H]NADPH but is so rapid with the 5-^{3}H-labeled dihydro-5-deazaflavins that stereochemical studies with that analogue were impractical (Fisher et al., 1976). The 8-hydroxy-5-deazaflavin system is just poised on the edge where one will be able to analyze stereochemical outcome of other F420-requiring reactions: reactions such as methanogen α-ketoacid reductive carboxylations (Zeikus et al., 1977) and methane production (Ellefson & Wolfe, 1980), as well as chlortetracycline biosynthesis in streptomycetes (Miller et al., 1960; McCormick & Morton, 1982).

Registry No. F420, 64885-97-8; FO, 37333-48-5; FOH$_2$, 76510-46-8; 8-OH-5-dFl$_{Et}$, 80547-90-6; 8-MeO-5-dFl$_{Et}$, 80547-91-7; 2-SH-RF, 17065-60-0; 7-Cl-RF, 7146-48-7; 8-Cl-RF, 5460-34-4; RF, 83-88-5; 1-dRF, 64183-66-0; 5-dRF, 19342-73-5; 8-OH-RF, 37163-36-3; 5-dRFH$_2$, 58865-23-9; hydrogenase (coenzyme F$_{420}$), 65099-08-3; coenzyme F$_{420}$–NADP reductase, 65099-09-4; NADPH-dependent FMN reductase, 56626-29-0; sulfite, 14265-45-3.

References

Adams, M. W., Mortenson, L. E., & Chen, J. S. (1981) *Biochim. Biophys. Acta 594*, 105.

Ashton, W. T., & Brown, R. D. (1980) *J. Heterocycl. Chem. 17*, 1709.

Ashton, W. T., Brown, R. D., Jacobson, F. S., & Walsh, C. T. (1979) *J. Am. Chem. Soc. 101*, 4419.

Balch, W. E., Magrum, L., Fox, G. E., Wolfe, R. S., & Woese, C. R. (1977) *J. Mol. Evol. 9*, 305.

Balch, W. E., Fox, G. E., Magrum, L. G., Woese, C. R., & Wolfe, R. S. (1979) *Microbiol. Rev. 43*, 260.

Becvar, J. (1973) Ph.D. Dissertation, University of Michigan, Ann Arbor, MI.

Benner, S. A. (1982) *Experientia 38*, 633.

Blankenhorn, G. (1976) *Eur. J. Biochem. 67*, 67.

Cheeseman, P., Toms-Wood, A., & Wolfe, R. S. (1972) *J. Bacteriol. 112*, 527.

Daniels, L. D. (1978) Ph.D. Dissertation, University of Wisconsin, Madison, WI.

Diekert, G., Gilles, H., Jaenchen, R., & Thauer, R. K. (1980a) *Arch. Microbiol. 128*, 256.

Diekert, G., Jaenchen, R., & Thauer, R. K. (1980b) *FEBS Lett. 119*, 118.

Diekert, G., Klee, B., & Thauer, R. K. (1980c) *Arch. Microbiol. 124*, 103.

Draper, R. D., & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802.

Duane, W., & Hastings, J. W. (1975) *Mol. Cell. Biochem. 6*, 53.

Edmondson, D., Barman, B., & Tollin, G. (1972) *Biochemistry 11*, 1133.

Eirich, L. D., Vogels, G. D., & Wolfe, R. S. (1978) *Biochemistry 17*, 4583.

Eirich, L. D., Vogels, G. D., & Wolfe, R. S. (1979) *J. Bacteriol. 140*, 20.

Eker, A. P. M. (1980) *Photochem. Photobiol. 32*, 593.

Eker, A. P. M., Pol, A., van der Myer, P., & Vogels, G. D. (1980) *FEMS Microbiol. Lett. 8*, 161.

Eker, A. P. M., Dekker, R. H., & Berends, W. (1981) *Photochem. Photobiol. 33*, 65.

Ellefson, W. L., & Wolfe, R. S. (1980) *J. Biol. Chem. 255*, 8388.

Ellefson, W. L., & Wolfe, R. S. (1981) *J. Biol. Chem. 256*, 4259.

Fisher, J. F. (1976) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.

Fisher, J., & Walsh, C. (1974) *J. Am. Chem. Soc. 96*, 4345.

Fisher, J., Spencer, R., & Walsh, C. (1976) *Biochemistry 15*, 1054.

Ghisla, S., & Mayhew, S. (1973) *J. Biol. Chem. 248*, 6568.

Ghisla, S., & Mayhew, S. (1976) *Eur. J. Biochem. 63*, 373.

Ghisla, S., & Mayhew, S. G. (1979) *Methods Enzymol. 63*, 241.

Ghisla, S., Massey, V., & Mayhew, S. G. (1976) in *Flavins and Flavoproteins, Proceedings of the International Symposium on Flavins and Flavoproteins, 5th, San Francisco* (Singer, T., Ed.) pp 334–342, Elsevier, Amsterdam.

Gunsalus, R. P., & Wolfe, R. S. (1978) *FEMS Microbiol. Lett. 3*, 191.

Harbury, H. A., LaNoue, K. F., Loach, P. A., & Amich, R. M. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 1708.

Hemmerich, P., Massey, V., & Fenner, H. (1977) *FEBS Lett. 84*, 5.

Jacobson, F. S. (1981) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.

Jacobson, F. S., Daniels, L. D., Fox, J. A., Walsh, C. T., & Orme-Johnson, W. H. (1982) *J. Biol. Chem. 257*, 3385.

Jencks, W. P., & Regenstein, J. (1976) in *Handbook of Biochemistry and Molecular Biology* (Fasman, G., Ed.) 3rd ed., CRC Press, Inc., Cleveland, OH.

Jorns, M., & Hersh, L. (1975) *J. Biol. Chem. 251*, 4872.
Kearney, E., Goldenberg, J., Lipsick, M., & Perl, M. (1979) *J. Biol. Chem. 254*, 9551.
Massey, V., Muller, F., Feldberg, R., Schuman, M., Sullivan, P., Howell, L., Mayhew, S., Matthews, R., & Foust, G. (1969) *J. Biol. Chem. 244*, 3999.
Mayhew, S. G. (1971a) *Biochim. Biophys. Acta 235*, 276.
Mayhew, S. G. (1971b) *Biochim. Biophys. Acta 235*, 289.
Mayhew, S. G. (1973) *Eur. J. Biochem. 85*, 535.
Mayhew, S. G., & Massey, V. (1969) *J. Biol. Chem. 244*, 794.
Mayhew, S. G., Whitfield, C. D., Ghisla, S., & Jorns, M. S. (1974) *Eur. J. Biochem. 44*, 579.
McCormick, J. R. D., & Morton, G. O. (1982) *J. Am. Chem. Soc. 104*, 4014.
McCormick, J. R. D., Sjolander, N. A., Miller, P. A., Hirsch, U., Arnold, N. H., & Doerschuk, A. P. (1958) *J. Am. Chem. Soc. 80*, 6460.
Miller, P. A., Sjolander, N. A., Nalesnyk, S., Arnold, N., Johnson, S., Doerschuk, A. P., & McCormick, J. R. D. (1960) *J. Am. Chem. Soc. 82*, 5002.
Muller, F., Brustlein, M., Hemmerich, P., Massey, V., & Walker, W. (1972) *Eur. J. Biochem. 25*, 573.
O'Brien, D., Weinstock, L., & Cheng, C. (1970) *J. Heterocycl. Chem. 7*, 99.
Pol, A., van der Drift, C., Vogels, G. D., Cuppen, T. J. H. M., & Laarhoven, W. H. (1980) *Biochem. Biophys. Res. Commun. 92*, 255.
Schneider, K., & Schlegel, H. G. (1978) *Biochem. Biophys. Res. Commun. 84*, 564.
Scola-Nagelscheider, G., & Hemmerich, P. (1976) *Eur. J. Biochem. 66*, 567.
Spencer, R. W. (1978) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.
Spencer, R., Fisher, J., & Walsh, C. (1976) *Biochemistry 15*, 1043.
Spencer, R., Fisher, J., & Walsh, C. (1977) *Biochemistry 16*, 3586.
Stankovich, M., & Massey, V. (1976) *Biochim. Biophys. Acta 452*, 335.
Stombaugh, N. A., Sundquist, J. E., Burris, R. H., & Orme-Johnson, W. H. (1976) *Biochemistry 15*, 2633.
Suelter, C. H., & Metzler, D. E. (1960) *Biochim. Biophys. Acta 44*, 23.
Summers, L. A. (1980) in *The Bipyridinium Herbicides*, p 58, Academic Press, New York.
Taylor, C. D., & Wolfe, R. S. (1974) *J. Biol. Chem. 249*, 4879.
Tzeng, S. F., Bryant, M. P., & Wolfe, R. S. (1975a) *J. Bacteriol. 121*, 192.
Tzeng, S. F., Wolfe, R. S., & Bryant, M. P. (1975b) *J. Bacteriol. 121*, 184.
Walsh, C., Fisher, J., Spencer, R., Graham Ashton, W., Brown, J., & Rogeers, E. (1978) *Biochemistry 17*, 1942.
Whitman, W. B., & Wolfe, R. S. (1980) *Biochem. Biophys. Res. Commun. 92*, 1196.
Woese, C. R., Magrum, L. G., & Fox, G. E. (1978) *J. Mol. Evol. 11*, 245.
Yamazaki, S., Tsai, L., Stadtman, T. C., Jacobson, F., & Walsh, C. (1980) *J. Biol. Chem. 255*, 9025.
Zeikus, J. G., Fuchs, G., Kenealy, W., & Thauer, R. K. (1977) *J. Bacteriol. 132*, 604.

Comparison of F_1's of Oxidative Phosphorylation from *Escherichia coli* and *Salmonella typhimurium* and Demonstration of Interchangeability of Their Subunits†

Shih-Yuan Hsu, Mototaka Senda,‡ Hiroshi Kanazawa, Tomofusa Tsuchiya, and Masamitsu Futai*

ABSTRACT: The peripheral membrane portion (SF_1) of proton-translocating ATPase of *Salmonella typhimurium* and its α, β, and γ subunits were purified and compared with the same portion (EF_1) from *Escherichia coli*. The α, β, and γ subunits of these F_1's were found to be mutually interchangeable, and all possible combinations of the three subunits from EF_1 and SF_1 showed ATPase activity. Both F_1's could bind functionally to the integral membrane part (F_0) of either bacterium, suggesting that F_0 and F_1 are interchangeable in these two bacteria and thus that the two F_1's are closely similar at the level of subunit structure. However, SF_1 differed from EF_1 in some enzymological properties such as its specific activity and susceptibilities to sodium dodecyl sulfate and methanol. The specific ATPase activity of EF_1 was more than twice that of SF_1, and hybrid enzymes containing the β subunit of EF_1 had higher activity than other hybrids. Amino acid analysis suggested that the primary structures of the α subunits of the two F_1's are less homologous than those of the β subunits. Thus, the primary structure of the α subunit may be more species specific than that of the β subunit.

The proton-translocating ATPases (F_0F_1)[1] in membranes of chloroplasts, mitochondria, and bacteria catalyze ATP synthesis coupled with electron transport [for reviews, see Futai & Kanazawa (1983), Maloney (1983), and Senior & Wise (1983)]. The catalytic portion of the enzyme, F_1, is composed of five different subunits (α, β, γ, δ, and ϵ), while the intrinsic membrane portion, F_0, which functions as a proton pathway

† From the Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700, Japan. *Received June 29, 1983.* This research was supported by grants from the Ministry of Education, Science and Culture of Japan and the Toray Science Foundation.

‡ Present address: Nippon Roche Research Center, Kamakura, Japan.

[1] Abbreviations: F_0, integral membrane portion of the proton-translocating ATPase; F_1, peripheral membrane portion of the proton-translocating ATPase; F_0F_1, entire proton-translocating ATPase; EF_1, F_1 from *E. coli*; TF_1, F_1 from the thermophilic bacterium PS3; SF_1, F_1 from *S. typhimurium*; $NaDodSO_4$, sodium dodecyl sulfate; α, β, γ, δ, and ϵ, subunits of F_1; a, b, and c, subunits of F_0; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

0006-2960/84/0423-0988$01.50/0