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Stereochemistry and Mechanism of Hydrogen Transfer between NADPH and Methylenetetrahydrofolate in the Reaction Catalyzed by Methylenetetrahydrofolate Reductase from Pig Liver

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Abstract: Methylenetetrahydrofolate reductase from pig liver is a flavoprotein that catalyzes the reduction of $N^{5,10}$-methylenetetrahydrofolate (CH_2-H_4folate) to N^5-methyltetrahydrofolate (CH_3-H_4folate) using reducing equivalents supplied by NADPH. In these studies, the physiological cofactor for methylenetetrahydrofolate reductase, flavin adenine dinucleotide, has been replaced by 8-demethyl-8-hydroxy-5-deaza-5-carbaflavin adenine dinucleotide hydroquinone (8-OH-5-deazaFADH$_2$). Using this flavin analogue, the stereochemistry of hydrogen transfer between the deazaflavin hydroquinone and both NADP$^+$ and CH_2-H_4folate has been determined. Tritium from (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ is incorporated quantitatively into (4*S*)-[4-^{3}H]NADPH or into the methyl group of CH_3-H_4folate. This is the first example of a flavoprotein that reacts with pyridine nucleotides on the *si*, rather than the *re*, face of 8-OH-5-deazaFADH$_2$. The *re* and *si* faces of the flavin isoalloxizine are defined relative to C^5 of the oxidized flavin. The observation that both NADP$^+$ and CH_2-H_4folate react at the same face of the enzyme-bound deazaflavin is consistent with the bi-bi ping-pong kinetics previously observed with this enzyme [Matthews, R. G.; Haywood, B. J. *Biochemistry* **1979**, *18*, 4845] and supports a mechanism for the NADPH-dependent reduction of CH_2-H_4folate in which NADP$^+$ dissociates before CH_2-H_4folate binds the reduced enzyme. In the presence of 8-OH-5-deazaFAD, methylenetetrahydrofolate reductase apoenzyme has a k_{cat} value for the NADPH-CH_2-H_4folate oxidoreductase reaction that is 52% of that observed for the apoenzyme assayed in the presence of FAD. These observations suggest hydride transfer mechanisms for the reduction of enzyme-bound flavin by NADPH and its reoxidation by CH_2-H_4folate.

Pig liver methylenetetrahydrofolate reductase catalyzes the transfer of reducing equivalents from NADPH to CH_2-H_4folate[1] to generate CH_3-H_4folate and NADP$^+$. Rapid reaction stopped-flow kinetic studies of individual half-reactions have established that the enzyme-bound cofactor, FAD, is reduced by NADPH and reoxidized by CH_2-H_4folate at rates that are consistent with the observed rate of turnover.[2] The reaction catalyzed by methylenetetrahydrofolate reductase can be described by eqs 1 and 2. Although formation of the flavin hydroquinone anion

$$\text{NADPH} + \text{E·FAD} \rightarrow \text{NADP}^+ + \text{E·FADH}^- \quad (1)$$

$$\text{E·FADH}^- + \text{CH}_2\text{-H}_4\text{folate} + \text{H}^+ \rightarrow \text{E·FAD} + \text{CH}_3\text{-H}_4\text{folate} \quad (2)$$

(FADH$^-$) is consistent with the overall stoichiometry of the reaction, the actual ionization state of the enzyme bound flavin hydroquinone has not been determined. Bi-bi ping-pong kinetics are observed for the NADPH-CH_2-H_4folate oxidoreductase reaction,[3] which are consistent with a kinetic mechanism in which NADP$^+$ release precedes the binding of CH_2-H_4folate.

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(1) The abbreviations used are as follows: CH_2-H_4folate, $N^{5,10}$-methylenetetrahydrofolate; CH_3-H_4folate, N^5-methyltetrahydrofolate; FAD, flavin adenine dinucleotide; 8-OH-5-deazaFAD, 8-demethyl-8-hydroxy-5-deaza-5-carbaflavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; AcPyAD$^+$, oxidized 3-acetylpyridine adenine dinucleotide; AcPyADP$^+$, oxidized 3-acetylpyridine adenine dinucleotide phosphate; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography.

(2) Vanoni, M. A.; Ballou, D. P.; Matthews, R. G. *J. Biol. Chem.* **1983**, *258*, 11 510–11 514.

(3) Matthews, R. G.; Haywood, B. J. *Biochemistry* **1979**, *18*, 4845–4851.

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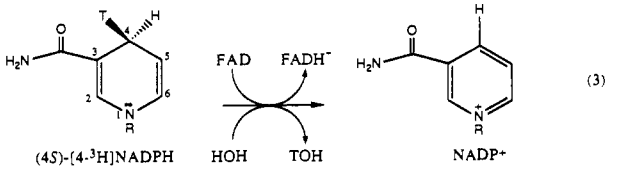
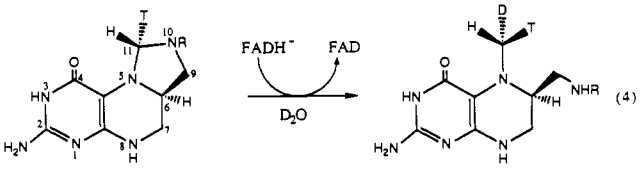
Previous studies with methylenetetrahydrofolate reductase have elucidated the substrate stereochemistry of hydrogen transfer from NADPH to flavin in the reductive half reaction, eq 1, and from flavin hydroquinone to CH_3-H_4folate in the oxidative half reaction, eq 2. As shown in eq 3, methylenetetrahydrofolate reductase stereospecifically removes tritium from (4*S*)-[4-^{3}H]NADPH and releases it to the solvent.[4] Presumably, the exchange of tritium with solvent occurs after transfer of the 4*S* tritium from NADPH to N^5 of the enzyme-bound flavin. The oxidative half reaction results in the incorporation of deuterium from solvent into the methyl group of CH_3-H_4folate,[5] and stereochemical analysis of this reaction has shown that (11*R*)-[11-^{3}H]CH_2-folate is converted to (*R-methyl*)CH_3-H_4folate during reduction in D_2O,[6] as shown in eq 4. Thus, the reduction of (11*R*)-[11-^{3}H]CH_2-H_4folate occurs with addition of deuterium to the more sterically accessible face of the pterin, i.e., the face opposite the bulky substituent at C^6 of the pterin ring. Determination of the complete stereochemistry of the NADPH-CH_2-H_4folate oxidoreductase reaction requires a knowledge of the orientation of each substrate with respect to the enzyme-bound flavin.

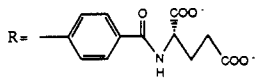


(4*S*)-[4-^{3}H]NADPH

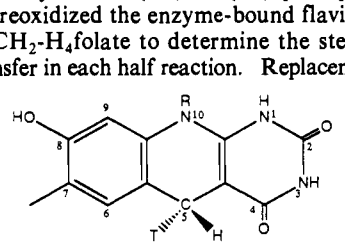
R = β-1'-ribosyl-5'-diphosphoadenosine-2'-phosphate



(11*R*)-[11-^{3}H]CH_2-H_4folate (*R-methyl*)CH_3-H_4folate



Manstein et al. have used 8-demethyl-8-hydroxy-5-deaza-5-carbaflavins to determine the stereochemistry of hydrogen transfer to and/or from the flavin of several flavoproteins.[7,8] These analyses are based on the demonstration that sodium [^{3}H]borohydride reduction of 8-OH-5-deaza-FAD bound at the active site of general acyl-CoA dehydrogenase results in the stereospecific synthesis of (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ (**1**).[7] In the present study, we have reconstituted methylenetetrahydrofolate reductase apoenzyme with (5*R*)- or (5*S*)-[5-^{3}H]-8-OH-5-deaza-FADH$_2$ and reoxidized the enzyme-bound flavin analogue with $NADP^+$ or CH_2-H_4folate to determine the stereochemistry of hydrogen transfer in each half reaction. Replacement of the flavin



(5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ (**1**)

of methylenetetrahydrofolate reductase with 8-OH-5-deazaFAD can also provide insight into the mechanism of enzymatic reduction of CH_2-H_4folate. Three possible modes of reduction of CH_2-H_4folate by methylenetetrahydrofolate reductase are shown in Scheme I. All of the reactions are postulated to proceed through a 5-iminium cation (**2**), for which evidence has been obtained in model studies of the condensation of H_4folate with formaldehyde.[9] Pathway A involves a direct hydride transfer from the reduced flavin to the exocyclic methylene group of the 5-iminium cation. Pathway B depicts an intramolecular rearrangement to generate 5-methyl-quinoid-H_2folate (**3**) followed by electron or hydride transfer from the reduced flavin to the pterin ring. Pathway C shows a one-electron reduction of the 5-iminium cation to form a methylene radical (**4**) followed by hydrogen atom transfer from the neutral flavin semiquinone to form CH_3-H_4folate.

Methylenetetrahydrofolate reductase is unusual among flavoproteins in that the enzyme fails to stabilize the flavin semiquinone. This species has not been observed during reductive titration with CH_3-H_4folate[10] or NADPH[11] or during reoxidation of photoreduced enzyme with CH_2-H_4folate.[11] One-electron pathways for reduction of CH_2-H_4folate have been postulated, however, because methylenetetrahydrofolate reductase readily reduces menadione[3,11,12] and quininoid dihydropterins,[13] including those lacking a substituent at the N^5 position. Reduction of the latter substrates necessarily involves introduction of reducing equivalents into the pterin ring. These studies were taken as evidence that reducing equivalents are also transferred from the reduced flavin into the pterin ring of CH_2-H_4folate as shown in pathway B.

Measurements of the catalytic activity of flavoproteins reconstituted with 5-deazaflavin analogues are mechanistically informative because 5-deazaflavins fail to stabilize the semiquinone oxidation state.[14] If reduction proceeds by hydride transfer to the 5-iminium cation formed by protonation of CH_2-H_4folate (pathway A), enzyme reconstituted with 8-OH-5-deazaFAD should retain at least partial activity and tritium should be incorporated into the methyl group of CH_3-H_4folate, whereas if reduction proceeds by pathway C, the reconstituted enzyme should be unable to reduce CH_2-H_4folate or should show greatly decreased catalytic activity. The mechanism shown in pathway B would lead to release of tritium to solvent from C^5 of reduced enzyme-bound deazaflavin, rather than transfer of tritium into the methyl group of CH_3-H_4folate.

Results

Stereochemistry of Hydrogen Transfer between 8-OH-5-deazaFADH$_2$ and CH_2-H_4folate. The apoenzyme of methylenetetrahydrofolate reductase was reconstituted with either (5*S*)- or (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$, stereospecifically synthesized as described in the Experimental Section. Enzyme-bound (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ was mixed with CH_2-H_4folate anaerobically, and reoxidation was monitored spectrally. The characteristic absorbance peak of oxidized free 8-OH-5-deaza-FAD,[7] with a maximum at 430 nm, appeared during reoxidation, indicating that ~20% of the bound deazaflavin hydroquinone reoxidized. The fraction of deazaflavin reoxidized was in good agreement with the 28% reconstitutable NADPH-menadione oxidoreductase activity seen for this preparation of apoenzyme after incubation with FAD. Upon concentration of the oxidized enzyme solution in a Centricon 30 microconcentrator (Amicon) we observed 25% of the tritium in the filtrate. Spectrally, we observed that the filtrate contained all of the oxidized deazaflavin, while the protein and deazaflavin hydroquinone remained in the concentrate. This indicated that the oxidized form of 8-OH-5-deazaFAD binds the protein less tightly than the reduced form. An aliquot of the filtrate was analyzed by reversed phase HPLC and the radioactivity of fractions was monitored by liquid scintillation counting. The tritium in the filtrate eluted with the same

(4) Vanoni, M. A.; Matthews, R. G. *Biochemistry* **1984**, *23*, 5272–5279.
(5) (a) Kisliuk, R. L. *J. Biol. Chem.* **1963**, *238*, 397–400. (b) Matthews, R. G. *Biochemistry* **1982**, *21*, 4165–4171.
(6) Vanoni, M. A.; Lee, S.; Floss, H. G.; Matthews, R. G. *J. Am. Chem. Soc.* **1990**, *112*, 3987–3992.
(7) Manstein, D. J.; Pai, E. F.; Schopfer, L. M.; Massey, V. *Biochemistry* **1986**, *25*, 6807–6816.
(8) Manstein, D. J.; Pai, E. F.; Schopfer, L. M.; Massey, V. *Biochemistry* **1988**, *27*, 2300–2305.
(9) Kallen, R. G.; Jencks, W. P. *J. Biol. Chem.* **1966**, *241*, 5851–5863.
(10) Daubner, S. C.; Matthews, R. G. *Flavins and Flavoproteins*; Massey, V., Williams, C. H., Eds.; Elsevier North Holland: Amsterdam, 1982; pp 165–172.
(11) Daubner, S. C.; Matthews, R. G. *J. Biol. Chem.* **1982**, *257*, 140–145.
(12) Kutzbach, C.; Stokstad, E. L. R. *Biochim. Biophys. Acta* **1971**, *250*, 459–477.
(13) Matthews, R. G.; Kaufman, S. *J. Biol. Chem.* **1980**, *255*, 6014–6017.
(14) (a) Bruice, T. C. *Prog. Bioorg. Chem.* **1976**, *4*, 1–87. (b) Hemmerich, P.; Massey, V.; Fenner, H. *FEBS Lett.* **1977**, *84*, 5–21. (c) Jacobson, F.; Walsh, C. *Biochemistry* **1984**, *23*, 979–988.

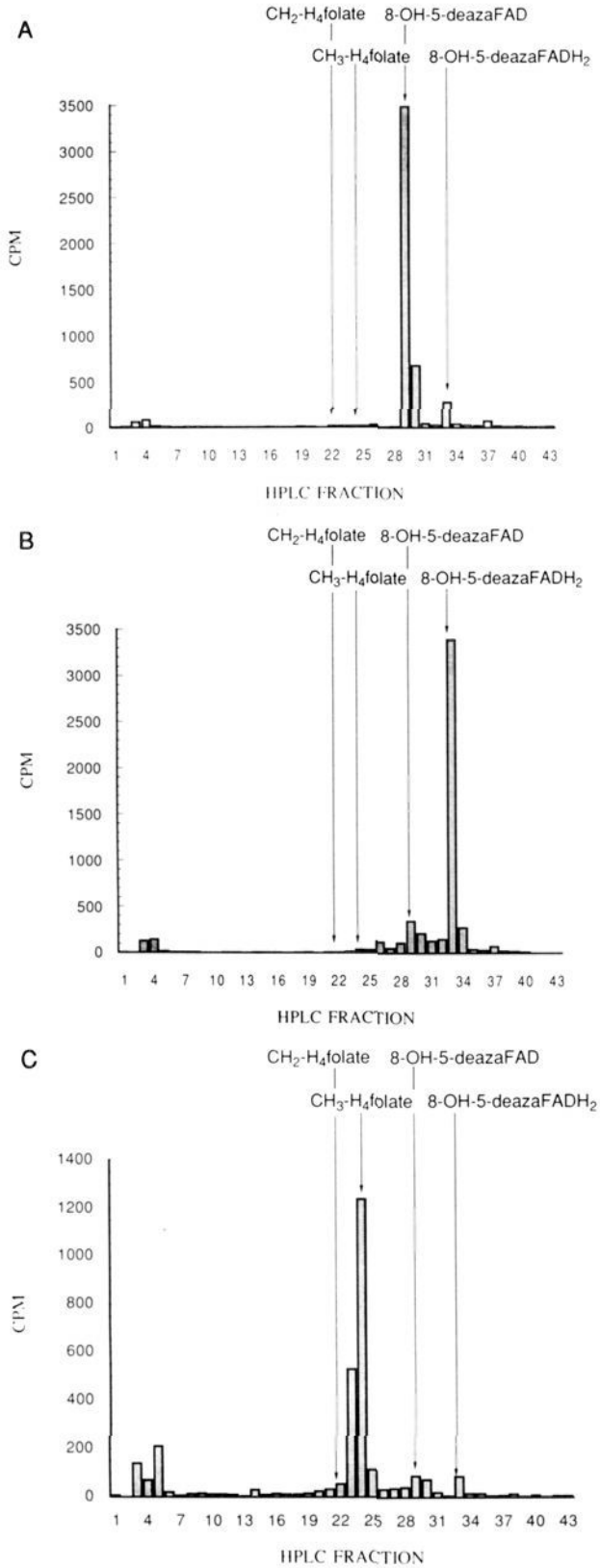


Figure 1. Analysis of reaction products formed by reoxidation of 8-OH-5-deazaflavin hydroquinone by CH_2-H_4folate. The positions of tritiated fractions eluting from reversed phase HPLC analysis are shown relative to the elution position of standards. One milliliter fractions were collected for analysis by liquid scintillation counting. (A) The filtrate obtained from the reoxidation of enzyme-bound (5*R*)-[5-^{3}H]-8-OH-5-deazaFAD with CH_2-H_4folate, (B) $NaBH_4$ reduction of the filtrate analyzed in (A), and (C) the reaction products formed from the reoxidation of enzyme-bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with CH_2-H_4folate.

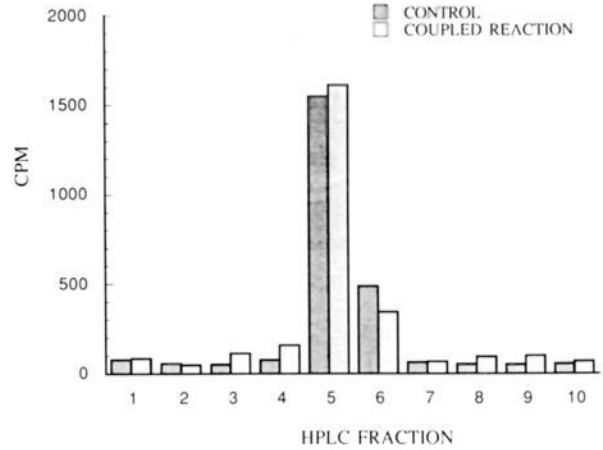


Figure 2. Product of the reoxidation of enzyme-bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with CH_2-H_4folate in the presence of methionine synthase and homocysteine. This figure illustrates the reversed phase HPLC analysis of the reaction products obtained when reoxidation of the enzyme-bound deazaflavin hydroquinone by CH_2-H_4folate is coupled to the transmethylation catalyzed by cobalamin-dependent methionine synthase. Methionine synthase transfers the methyl group of CH_3-H_4-folate to homocysteine to generate methione and H_4folate. The control illustrates the elution profile of a reaction in which 5-[*methyl*-^{14}C]-CH_3-H_4folate is incubated with homocysteine and methionine synthase and then analyzed by HPLC. Details of the procedure are described in the Experimental Section.

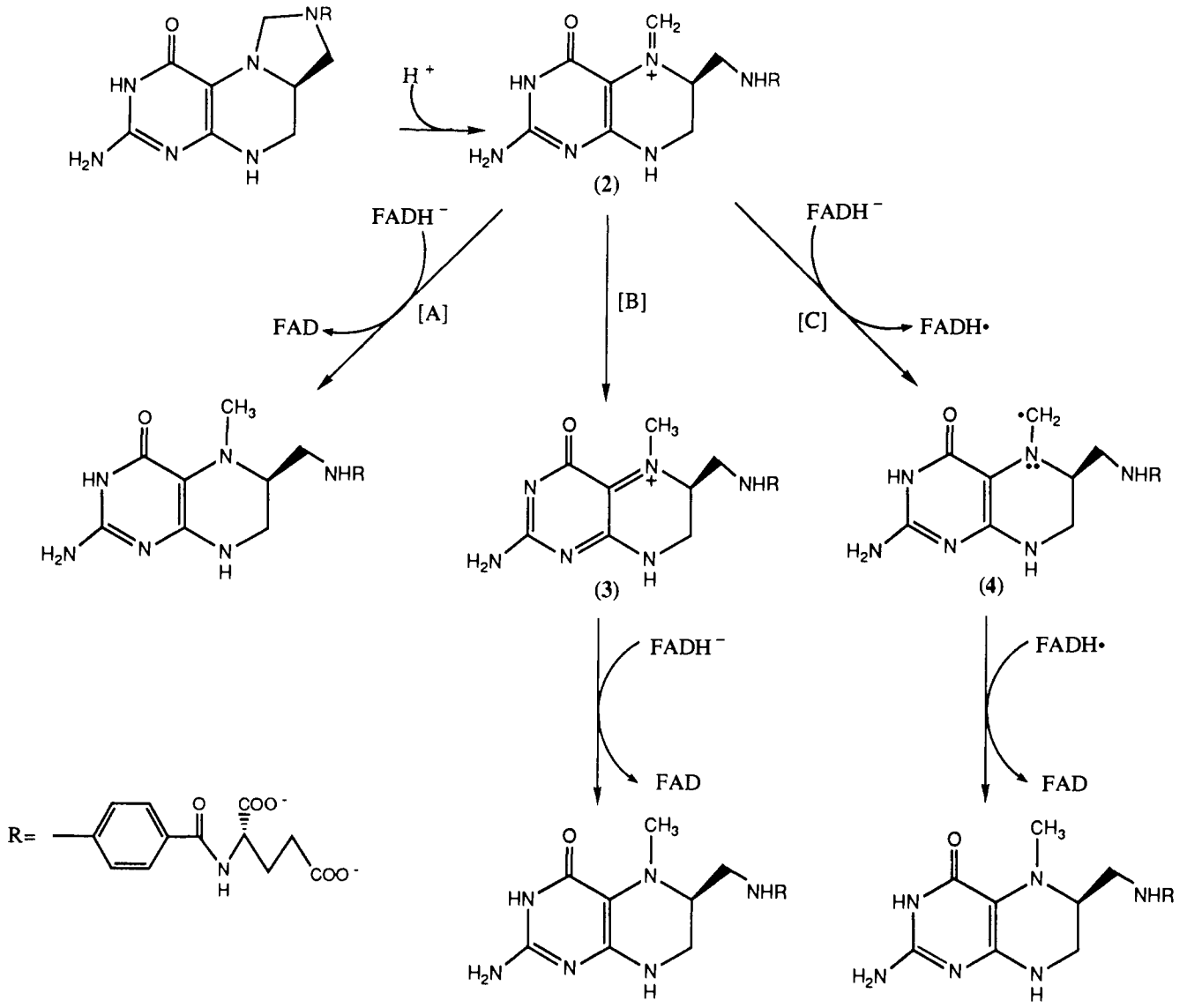
retention time as 8-OH-5-deazaFAD, as shown in Figure 1A. To confirm that the tritium was associated with 8-OH-5-deazaFAD, a second aliquot of the filtrate was mixed with $NaBH_4$ and analyzed by HPLC. As shown in Figure 1B, the tritium now eluted with the same retention time as 8-OH-5-deazaFADH$_2$. These results indicate that there is no transfer of tritium from enzyme-bound (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ during oxidation with CH_2-H_4folate.

In a similar manner, approximately 74 pmol of enzyme-bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ was oxidized anaerobically by mixing with CH_2-H_4folate and the tritiated product was analyzed by HPLC. Before the addition of 15 nmol of CH_2-H_4folate, 4 nmol of unlabeled CH_3-H_4folate was added anaerobically to reduce any remaining enzyme-bound FAD to the hydroquinone form and to dilute any labeled CH_3-H_4folate formed during reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ by CH_2-H_4folate. CH_2-H_4folate and CH_3-H_4folate are in reversible equilibrium with the oxidized and hydroquinone forms of enzyme-bound FAD. The midpoint potential for the enzyme-bound FAD/FADH$_2$ couple, −143 mV vs the standard hydrogen electrode at pH 7.2, where these experiments were performed, is only 10 mV more negative than the CH_2-H_4folate/CH_3-H_4folate couple at the same pH value.[4,15] The reoxidized enzyme sample was injected directly onto the HPLC column, without concentration in a microconcentrator. 73% of the total tritium in the sample coeluted with CH_3-H_4folate, and no other tritiated peaks were observed, as shown in Figure 1C. Presumably any residual reduced deazaflavin remains bound to the enzyme and is retained on the HPLC column.

To verify the identity of the tritiated species and to determine whether tritium was transferred to the methyl group of CH_3-H_4folate, the reoxidation was repeated in the presence of methionine synthase and homocysteine. Methionine synthase transfers the methyl group of CH_3-H_4folate to homocysteine to generate methionine. Therefore, if tritium were in the methyl group of CH_3-H_4folate after reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ by CH_2-H_4folate, then tritium should be found in methionine. HPLC analysis after the coupled reaction showed that the tritiated species eluted at approximately 5.5 min (Figure 2), which is the same retention time as seen for methionine. These

(15) Katzen, H. M.; Buchanan, J. M. *J. Biol. Chem.* **1965**, *240*, 825–835.

Scheme I



results indicate that CH_2-H_4folate binds on the *si* side of the enzyme-bound prosthetic group and tritium is transferred from C^5 of the reduced deazaflavin to the exocyclic methylene group of CH_2-H_4folate. The *si* and *re* faces of the flavin isoalloxizine ring are defined relative to N^5 of the oxidized flavin.

Stereochemistry of Hydrogen Transfer between 8-OH-5-deazaFADH$_2$ and NADP$^+$. The reductive half reaction of methylenetetrahydrofolate reducase shown in eq 1 is irreversible when FAD is the enzyme-bound cofactor, because binding of FAD to the apoenzyme shifts the midpoint potential at pH 7.2 for the FAD/FADH$^-$ couple from −231[16] to −143 mV vs SHE,[4] while the midpoint potential at pH 7.2 for the NADP$^+$/NADPH couple is −335 mV.[17] 7,8-Didemethyl-8-hydroxy-5-deaza-5-carbariboflavin, a very close analogue of 8-OH-5-deazaflavin, has a midpoint potential for the oxidized/hydroquinone couple at pH 7.0 of −350 mV,[14c] which is 119 mV lower than that for FAD/FADH$^-$. Consequently, when 8-OH-5-deaza-FADH$_2$ is bound to methylenetetrahydrofolate reductase, the potential is low enough to permit substantial reduction of NADP$^+$.

To determine which face of the deazaflavin cofactor interacts with NADP$^+$, either (5*S*)- or (5*R*)-[5-^{3}H]-8-OH-5-deaza-FADH$_2$ was bound to methylenetetrahydrofolate reductase apoenzyme and reoxidized with NADP$^+$. The apoenzyme recovered from the reoxidation of (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with CH_2-H_4folate was reconstituted with (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$. NADP$^+$ was added, and the protein solution was concentrated in a Centricon 30 microconcentrator after the reoxidation. The filtrate contained 9% of the tritium, indicating approximately 9% reoxidation. This low extent of reoxidation is probably due to reuse of the apoenzyme in this experiment. The filtrate was analyzed by reversed phase HPLC and the tritiated fraction eluted with the same retention as 8-OH-5-deazaFAD, as shown in Figure 3A. That is, when (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ is oxidized with NADP$^+$, there is no transfer of tritium to NADPH.

Reoxidation of approximately 74 pmol of enzyme-bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ by NADP$^+$ was performed under anaerobic conditions and directly analyzed by HPLC. Before the addition of 225 nmol of NADP$^+$, 10 nmol of NADPH was added anaerobically to irreversibly reduce any residual enzyme-bound FAD. After the addition of NADP$^+$ and HPLC analysis, the tritiated fraction had the same retention time as NADPH (Figure 3B). Eighty-two percent of the tritium in the sample applied to the HPLC eluted in the NADPH fraction. Presumably any residual reduced deazaflavin remained enzyme-bound and was retained on the HPLC column.

Under aerobic conditions, residual enzyme-bound FAD in the apoenzyme preparation is able to oxidize stereospecifically any labeled NADPH made upon reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with NADP$^+$. That is, if the label is transferred

(16) Clark, W. M. *Oxidation-Reduction Potentials of Organic Systems*; Williams and Wilkins: Baltimore, 1960; p 444.
(17) Engel, P. C.; Dalziel, K. *Biochem. J.* **1967**, *105*, 691–695.

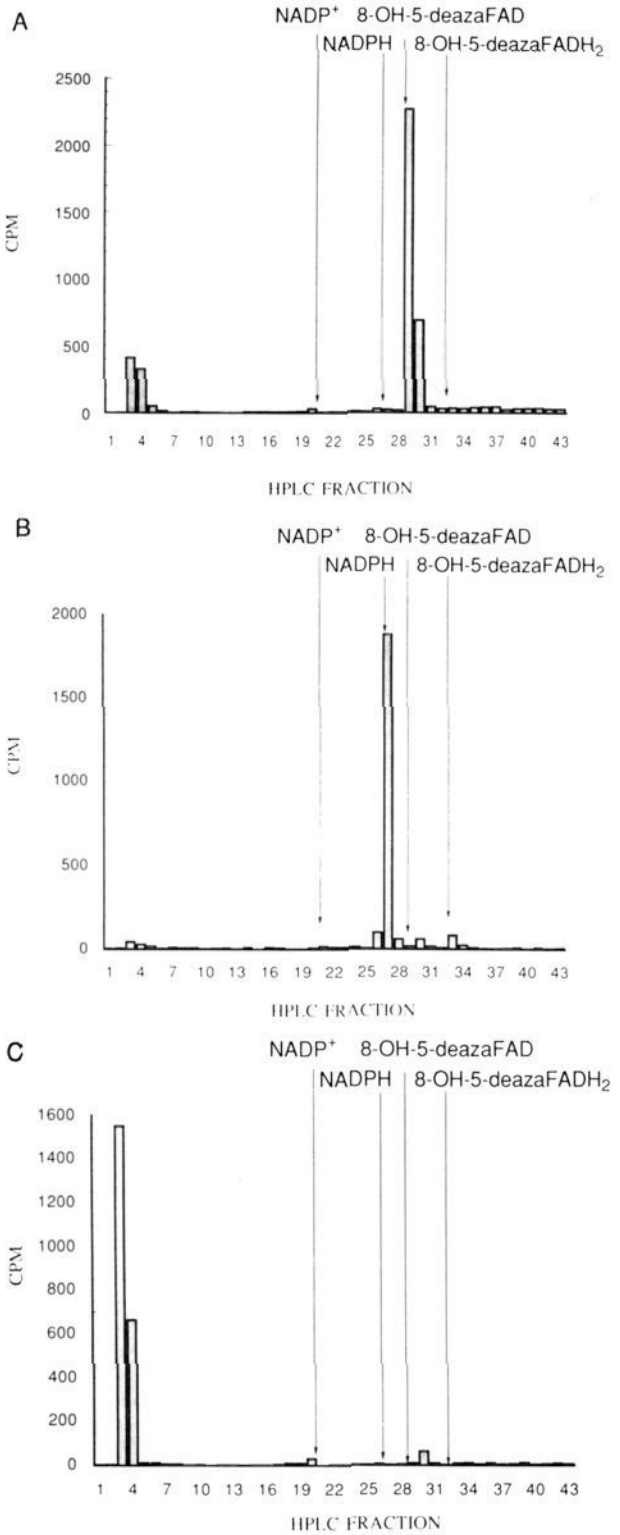


Figure 3. Products of the reoxidation of enzyme-bound [5-^{3}H]-8-OH-5-deazaFADH_2 by NADP$^+$. (A) HPLC analysis of the reoxidation of enzyme-bound (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH_2 by NADP$^+$, (B) HPLC analysis of the reoxidation of enzyme-bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH_2 by NADP$^+$ under anaerobic conditions; and (C) HPLC analysis of the reoxidation of enzyme-bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH_2 by NADP$^+$ under aerobic conditions.

to the 4*S* position of NADPH, then upon reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH_2 residual enzyme-bound FAD will catalyze the removal of tritium from the 4*S* position of NADPH. Because enzyme-bound FADH_2 rapidly reoxidizes aerobically, tritium is then exchanged into the solvent. If reoxidation of deazaflavin hydroquinone places tritium either randomly on NADP$^+$ or in the 4*R* position, then some or all of the tritium will end up in NADP$^+$ when the labeled NADPH is oxidized by FAD. To further identify the tritiated fraction as NADPH and determine whether the tritium was transferred stereospecifically to NADP$^+$, the reoxidation was repeated aerobically without the prior addition of NADPH. All the tritium eluted from the HPLC at the void volume (Figure 3C). This observation established that tritium is stereospecifically transferred from the *si* side of the flavin to the 4*S* position of NADPH.

Oxidoreductase Activity Measurements of Methylenetetrahydrofolate Reductase Reconstituted with 8-OH-5-deazaflavin. Three assays were used to measure the oxidoreductase activity of methylenetetrahydrofolate reductase in the presence of 8-OH-5-deazaFAD: the NADPH-CH_2-H_4folate oxidoreductase assay,[3] the NADPH-menadione oxidoreductase assay,[18] and the NADPH-quinoid-H_2folate oxidoreductase assay.[13] Because the oxidized 8-OH-5-deazaflavin does not bind tightly to methylenetetrahydrofolate reductase, holoenzyme and apoenzyme were incubated with an excess of deazaflavin or FAD. Table I shows oxidoreductase specific activities for the three assays. Preincubation of the holoenzyme with FAD increased all activities slightly, whereas preincubation of holoenzyme with 8-OH-5-deazaFAD led to small decreases in activity of the holoenzyme. The apoenzyme activity in the absence of any additional flavin indicated the presence of a small amount of residual FAD. After incubation of the apoenzyme with FAD, the NADPH-CH_2-H_4folate oxidoreductase activity is increased ~15-fold. Preincubation with 8-OH-5-deazaFAD increases the activity of the apoenzyme only 6% in the NADPH-menadione oxidoreductase assay and 4% in the NADPH-quinoid-H_2folate oxidoreductase assay. Thus there is almost no increase in activity when apoenzyme is incubated with 8-OH-5-deazaFAD when the electron acceptor is either menadione or quinoid-H_2folate. However, there is a 7-fold increase in activity in the NADPH-CH_2-H_4folate oxidoreductase assay when apoenzyme is preincubated with 8-OH-5-deazaFAD. This large increase, 52% of the increase in activity seen on incubation of apoenzyme with FAD, indicates that hydride transfer from enzyme-bound deazaflavin hydroquinone to CH_2-H_4folate proceeds in a kinetically competent fashion.

Discussion

Flavin analogues have proven very useful in probing the active sites of numerous flavoproteins.[19] 8-OH-5-deazaflavins have particularly useful properties for the determination of the stereochemistry of hydrogen transfer to and from the flavin. Reduced 8-OH-5-deazaflavins are only sluggishly reactive with oxygen and therefore can be handled in aerobic solutions.[14c,7] The hydroquinone can be formed by stereoselective reduction,[7] and the exchange of hydride between oxidized and hydroquinone species is markedly slower than the comparable rate of exchange of 5-deazaflavins,[14c] permitting stereochemical analysis.[20,7,8] Because reduction of 8-OH-5-deazaflavins and 5-deazaflavins leads to the incorporation of a hydride equivalent into a nonexchangeable position on the deazaflavin, the regiospecificity of hydride transfer from deazaflavin hydroquinone to the substrate can be determined.[7,8,14c,21] A stereospecific synthetic route to the (5*R*)-[5-^{3}H]-8-OH-5-deazaflavin hydroquinone has been established,[7] and in this study we report a method of generating the 5*S* diastereomer with high stereospecificity. The 5-deazaflavins appear to be reasonably good steric mimics of the natural flavin cofactors. When the flavin in glutathione reductase was replaced with 5-

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Table I. Specific Activities of Methylenetetrahydrofolate Reductase Apo- and Holoenzyme

activity measured (μmol NADPH mg^{-1} min^{-1})	no additions	+ FAD	+ 8-OH-5-deazaFAD
	NADPH-CH_2-H_4folate Oxidoreductase		
holoenzyme	1.84 ± 0.23 (91.0)[a]	2.03 ± 0.07 (100.0)	1.82 ± 0.12 (89.9)
apoenzyme	0.08 ± 0.009 (6.9)	1.13 ± 0.14 (100.0)	0.63 ± 0.07 (52.4)[b]
	NADPH-Menadione Oxidoreductase		
holoenzyme	10.07 ± 0.023 (83.7)	12.04 ± 0.17 (100.0)	10.18 ± 0.52 (84.6)
apoenzyme	1.59 ± 0.14 (13.2)	12.05 ± 0.57 (100.0)	2.23 ± 0.15 (6.1)[c]
	NADPH-Quinoid-H_2folate Oxidoreductase		
holoenzyme	0.62 ± 0.040 (93.9)	0.66 ± 0.087 (100.0)	0.55 ± 0.083 (83.3)
apoenzyme	0.07 ± 0.0064 (13.6)	0.49 ± 0.090 (100.0)	0.08 ± 0.015 (4.5)[c]

[a] The numbers in parentheses are the percent activity relative to the same enzyme preparation analyzed in the presence of FAD. [b] Corrected for residual holoenzyme activity: [(0.63 − 0.08)/(1.13 − 0.08)](100) = 52%. [c] Corrected for residual holoenzyme activity.

Table II. Summary of Studies of Flavoprotein Stereochemistry

enzyme	reductant/oxidant	face of flavin contacted	ref
glutathione reductase	NADPH	*re*	23a, 7
	redox active disulfide	*si*	
lipoamide dehydrogenase	reduced disulfide	*si*	23b
	NAD^+	*re*	
mercuric reductase	NADPH	*re*	7, 23c
	redox active disulfide	*si*	
thioredoxin reductase	NADPH	*re*	7, 23d
	redox active disulfide	*re*	
p-hydroxybenzoate hydroxylase	NADPH	*re*	7
melilotate hydroxylase	NADH	*re*	7
anthranilate hydroxylase	NADPH	*re*	7
2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase	$AcPyAD^+$	*re*	8
cyclohexanone monooxygenase	$AcPyADP^+$	*re*	8
NADH-FMN oxidoreductase	NADH	*re*	20
ferredoxin-$NADP^+$ oxidoreductase	$NADP^+$	*re*	23f
phthalate dioxygenase reductase	NADPH	*re*	23g
general acyl-CoA dehydrogenase	Acyl CoA	*re*	7, 31a
	$NaBH_4$	*re*	7
p-cresol methylhydroxylase	*p*-cresol	*re*	31b
glucose oxidase	glucose	*re*	7
D-amino acid oxidase	pyruvate, NH_3^+	*re*	8
glycolate oxidase	glycolate	*si*	31c
flavocytochrome b_2	pyruvate	*si*	31d
L-lactate oxidase	pyruvate	*si*	8
D-lactate dehydrogenase	pyruvate	*si*	8

deazaFAD, the X-ray diffraction pattern was not discernably different from that of the native enzyme.[22]

There are now a considerable number of flavoproteins for which the stereochemistry of transfer to and/or from the enzyme-bound flavin has been elucidated. Table II summarizes the results of determinations of the stereochemistry of hydrogen transfer to and/or from the flavin cofactor in a wide variety of flavoproteins. These results were obtained by stereochemical analysis of hydrogen transfer to and/or from enzymes reconstituted with 8-OH-5-deazaflavin or 7-demethyl-8-OH-5-deazaflavin cofactors or by X-ray crystallography of the native flavoproteins. As shown in Table II, 12 flavoproteins have been shown to react with pyridine nucleotide substrates at the *re* face of the flavin,[7,8,20,21] and, until the present study, none had been shown to interact with pyridine nucleotide at the *si* face of the flavin. As with pyridine nucleotides, both faces of the flavin are capable of transferring reducing equivalents to and/or from substrates (Table II). Benner has suggested that the two faces of reduced pyridine nucleotides are functionally distinct.[24] Functional distinctions between the *re* and *si* faces of enzyme-bound flavins have not yet been established. Thus far methylenetetrahydrofolate reductase is unique in reacting with its pyridine nucleotide substrate at the *si* face of the flavin, although several F_{420} dependent enzymes interact with pyridine nucleotides at the *si* face of this 7,8-didemethyl-8-OH-5-deazaflavin cofactor.[20]

There are two well established structural models for the interaction of an enzyme-bound flavin cofactor with two different substrates. Glutathione reductase, for which the X-ray structure is known, contains a flavin that divides the active site and reacts with pyridine nucleotide on its *re* face and with the redox active disulfide on its *si* face.[23a] In contrast, thioredoxin reductase reacts with both pyridine nucleotide and the redox active disulfide at the *re* face.[23d] Methylenetetrahydrofolate reductase presents still another pattern, reacting with both NADPH and CH_2-H_4folate at the *si* face.

Methylenetetrahydrofolate reductase is, to our knowledge, the first flavoprotein with a redox active flavin to exhibit a substantial fraction of the catalytic activity of the native enzyme when reconstituted with a 5-deazaflavin analogue, despite extensive studies of other flavoproteins reconstituted with either 5-deazaflavin or 8-OH-5-deazaflavin cofactors. As discussed by Hemmerich et al.[14b] and Bruice,[14a] 5-deazaflavins are thought to be unable to react with oxygen or to donate/accept single electrons because the semiquinone oxidation state is destabilized. The flavoproteins that catalyze reduction of molecular oxygen (oxidases) or hydroxylation (monoxygenases) have indeed been shown to be in-

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active when reconstituted with 5-deazaflavin analogues.[21e] The pyridine nucleotide disulfide oxidoreductases are also inactive when reconstituted with 5-deazaflavins, although they exhibit transhydrogenase activity. Flavodoxin is the only electron transferase to have been reconstituted with 5-deazaflavins, and it can no longer be reduced by NADPH in the presence of ferredoxin-NADPH reductase.[14b]

The model studies of Bruice and co-workers[21a,b] predict that dehydrogenases which function exclusively by transferring the elements of hydride from one substrate to another should be active when reconstituted with 5-deazaflavins. The studies of Jorns and Hersh[21c] have shown that *N*-methylglutamine synthetase reconstituted with 5-deazaFMN shows ~3% of the activity seen with native enzyme. The relatively low activity of the reconstituted enzyme may relate to the lower redox potential of the 5-deazaflavin cofactor as compared to FMN ($E^{\circ\prime}$ is −279[25] vs −231 mV[16]), since for native *N*-methylglutamine synthase, reoxidation of reduced glutaryl enzyme by methylamine is the rate limiting half reaction, while the enzyme reconstituted with 5-deazaflavin is limited in turnover by the rate of reduction of the enzyme by glutamate.

$$E_{ox} + \text{glutamate} \rightarrow E_{red}\text{-glutaryl} + NH_3$$

$$E_{red}\text{-glutaryl} + CH_3NH_2 \rightarrow E_{ox} + N\text{-methylglutamate}$$

A number of flavoprotein dehydrogenases have been reconstituted with deazaflavins, and their activities have been found to be reduced by factors of 100 to 10000.[14b] In these cases, the lower redox potential of the deazaflavin cofactor may have contributed to the decrease in activity observed.

In the case of methylenetetrahydrofolate reductase, the overall reaction proceeds with a free energy decrease of 9.5 kcal/mol, which is almost entirely associated with the reduction of the enzyme by NADPH (eq 1).[4] That is to say, binding of FAD to the apoenzyme raises the potential of the enzyme-bound flavin from −231[16] to −143 mV[4] or to nearly at the same potential as the CH_2-H_4folate/CH_3-H_4folate couple. Thus, despite the lower potential of the 8-OH-5-deazaFAD cofactor, if we assume a similar shift upwards in the potential of the analogue on binding to apoenzyme, as evidenced by weaker binding of oxidized 8-OH-5-deazaFAD compared with the hydroquinone form, reduction by NADPH should remain facile.

The observation of nearly comparable catalytic activity in methylenetetrahydrofolate reductase reconstituted with 8-OH-5-deazaFADH_2, as compared to native enzyme, also strongly favors pathway A in Scheme I as the mechanism of reduction of CH_2-H_4folate. First, we have demonstrated direct transfer of tritium from C^5 of the reduced deazaflavin to the methyl group of CH_3-H_4folate. This precludes reduction by pathway B, which postulates tautomerization of the 5-iminium cation (**2**) to form 5-methyl-quinoid-H_2folate (**3**) and subsequent reduction of this intermediate to form CH_3-H_4folate. Second, given the demonstrated unreactivity of the reduced 5-deazaflavins toward oxygen and other obligate one-electron acceptors.[14c,7] reduction of CH_2-H_4folate by a pathway such as C, which involves sequential electron and hydrogen atom transfers, should at the very least lead to greatly decreased catalytic activity in 8-OH-5-deazaflavin reconstituted enzyme. Our observation that the rates of reduction of menadione and quinoid-H_2folate are very substantially reduced in the deazaflavin reconstituted enzyme may suggest that reduction of these substrates normally involves one-electron transfers.

In summary, our studies have utilized several properties of 8-OH-5-deazaflavin to investigate the stereochemistry and mechanism of methylenetetrahydrofolate reductase. We have shown that both folate and pyridine nucleotide substrates react at the *si* face of the deazaflavin hydroquinone, in agreement with the ping-pong kinetics observed for the native enzyme.[3] Methylenetetrahydrofolate reductase thus far is unique among flavoproteins in binding a pyridine nucleotide substrate at the *si* face of the flavin. We have also shown that methylenetetrahydrofolate reductase reconstituted with 8-OH-5-deazaflavin retains ~50% of the activity of enzyme reconstituted with FAD. Since flavoproteins reconstituted with 5-deazaflavin analogues are generally unable to catalyze one-electron transfers,[14a,b] we conclude that the reaction catalyzed by methylenetetrahydrofolate reductase normally involves successive hydride transfers to and from the flavin cofactor. In the first step, NADPH binds at the *si* face of the flavin, and hydride is transferred from the 4 pro-*S* position of NADPH to the N^5 of the flavin. $NADP^+$ then dissociates from the reduced enzyme. In the second half of the reaction, CH_2-H_4folate binds at the *si* face of the reduced flavin and undergoes ring opening and protonation to form the 5-iminium cation. Hydride is then transferred from the N^5 position of the flavin to the exocyclic methylene group of the 5-iminium cation to form CH_3-H_4folate.

Experimental Section

Materials. Tritiated $NaBH_4$ (490 mCi/mmol) was purchased from NEN Research Products. 8-OH-5-deazaFAD was a generous gift from Dr. Vincent Massey and had been synthesized as previously described.[7] All other chemicals were of the finest grade possible and used without further purification.

Preparation and Storage of Enzymes. Methylenetetrahydrofolate reductase from pig liver was purified as previously described[18] and stored at −80 °C in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA and 10% glycerol. General acyl-CoA dehydrogenase was purified from pig kidney as described by Thorpe[26] with some modifications. The DEAE-cellulose column and subsequent purification steps were replaced by FPLC chromatography using a Pharmacia MonoQ 10/10 column. The column was eluted at 3 mL/min with a linear gradient from 50 to 500 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA. The specific activity determined after the FPLC was 2.73 μmol·mg^{-1} min^{-1} and the protein was ~90% homogeneous as visualized on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme was stored in the elution buffer at −80 °C. Cobalamin-dependent methionine synthase from *Escherichia coli* was prepared and stored as previously reported.[27]

Preparation of the Apoenzymes of Methylenetetrahydrofolate Reductase and General Acyl-CoA Dehydrogenase. Flavin was removed from methylenetetrahydrofolate reductase by mixing the holoprotein (25–40 nmol) with 2 M KBr in 50 mM potassium phosphate buffer, pH 5.9, containing 0.3 mM EDTA. The apoenzyme was separated from flavin by rinsing in a Centricon 30 microconcentrator with aliquots of 2 M KBr in 50 mM potassium phosphate buffer, pH 5.9, containing 0.3 mM EDTA. Rinsing was continued until there was no detectable flavin absorbance at 450 nm in the retained protein and little detectable NADPH-menadione oxidoreductase activity. It was occasionally necessary to dialyze the apoenzyme overnight in the KBr solution. The apoenzyme was then rinsed several times in the microconcentrator with 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA to remove the KBr. Glycerol was added to the sample to a final concentration of 10% for storage at −80 °C. The apoenzyme of general acyl-CoA dehydrogenase from pig kidney was freshly prepared by the large scale charcoal resolution method of Mayer and Thorpe.[28]

Reconstitution of General Acyl-CoA Dehydrogenase with the 8-OH-5-deazaFAD and Stereospecific Reduction. General acyl-CoA dehydrogenase apoprotein was reconstituted by a method of Manstein et al.[7] except that unbound flavin was removed using a Centricon 30 microconcentrator. Tritium was introduced stereospecifically into the *re* side of 8-OH-5-deazaFAD with tritiated $NaBH_4$ as previously described.[7] For the synthesis of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH_2, general acyl-CoA dehydrogenase was first reconstituted with [5-^{3}H]-8-OH-5-deazaFAD (see below) and then reduced with $NaBH_4$. Reduced deazaflavin was released from acyl CoA dehydrogenase by heat denaturation of the protein.[7]

Reconstitution of Methylenetetrahydrofolate Reductase with (5*R*)- or (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH_2. The apoenzyme of methylenetetrahydrofolate reductase was mixed with a stoichiometric amount of the reduced flavin analogue on ice for 3 to 18 h. The reconstituted holoenzyme was rinsed on a Centricon 30 microconcentrator several times with excess buffer (50 mM potassium phosphate containing 0.3 mM EDTA) until unbound flavin was removed, as judged by the absence of tritium in the filtrate. The reconstituted enzyme was stable to freezing at −80 °C if stored in 10% glycerol.

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Preparation of [5-^{3}H]-8-OH-5-deazaFAD. 8-OH-5-deazaFAD (21.3 nmol) in a 0.7-mL volume was incubated with 5 mCi of tritiated $NaBH_4$ for 30 min at 37 °C. Excess sodium pyruvate was added to convert any unreacted $NaBH_4$ to lactate. After reduction, 6% residual oxidized deazaflavin was observed spectrally. The racemic mixture was lyophilized overnight. During the workup, the reduced deazaflavin undergoes slow oxidation. Oxidized [5-^{3}H]-8-OH-5-deazaFAD was purified from tritiated lactate by HPLC using the protocol for separation of reoxidation products described below. Since no reduced flavin was observed on HPLC, the assumption was made that the postreduction manipulations allowed the flavin to reoxidize completely.

Reoxidation of Enzyme-Bound (5*R*)- or (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$. All of the reoxidation reactions were performed anaerobically under an argon atmosphere unless otherwise stated. The reoxidation of approximately 5 nmol of enzyme bound (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ by CH_2-H_4folate was followed spectrally in an anaerobic cuvette. Methylenetetrahydrofolate (10.5 nmol) was added with a Hamilton syringe. The reoxidized sample was concentrated in a Centricon 30 microconcentrator, which separates apoenzyme from oxidized deazaflavin and folate derivatives. To stabilize CH_3-H_4folate, dithiothreitol was added to the filtrate to give a final concentration of 50 mM. An aliquot of the filtrate was injected onto an HPLC column for analysis. The concentrate from this reoxidation was reconstituted with an excess of (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ and the unbound flavin was removed using a Centricon 30 microconcentrator. The reoxidation of the reconstituted reduced enzyme with $NADP^+$ (225 nmol) was performed in the same manner as with CH_2-H_4folate except that the procedure was performed under aerobic conditions and no dithiothreitol was added to the filtrate.

Analysis of the products of reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ was accomplished by injecting the reaction mixture directly onto the HPLC, bypassing the microconcentrator filtration step. This allowed the use of smaller amounts of reconstituted deazaflavin (74 pmol) and avoidance of prolonged exposure to aerobic conditions. Before the reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with $NADP^+$, 10 nmol of NADPH was added to reduce any residual native enzyme and to serve as a carrier for recovery of labeled NADPH, and, before reoxidation with CH_2-H_4folate, 4 nmol of CH_3-H_4folate was added. After a 10-min incubation on ice, $NADP^+$ (225 nmol) or CH_2-H_4folate (15 nmol) was added for the respective reoxidation. All of the reoxidation mixture was injected onto the HPLC after a 10-min incubation.

Analytical Separation of the Reoxidation Components. Oxidation products were separated on a Beckman HPLC system with a 5 μ Ultrasphere ODS column (4.5 mm × 25 cm) using a method adapted from Green et al.[29] Samples were injected onto a column that was preequilibrated with 95% 5 mM tetrabutylammonium phosphate (Pic A, Waters) and 5% isopropyl alcohol. The column was eluted at 1 mL/min with 5% isopropyl alcohol for 6 min and then with a 30-min linear gradient from 5 to 25% isopropyl alcohol. The absorbance was monitored at 254 nm and 1-mL fractions were collected. Retention times were determined for each of the products by comparison with authentic samples. The standard retention times are as follows: $NADP^+$, 20 min; CH_2-H_4folate, 22 min; CH_3-H_4folate, 24 min; NADPH, 27 min; 8-OH-5-deazaFAD, 29 min; and 8-OH-5-deazaFADH$_2$, 33 min. The elution profile of 5-[*methyl*-^{14}C]CH_3-H_4folate showed a delay of 1 min between the detection of the absorbance peak and elution of radioactivity, and the reported elution times for radiolabeled fractions have been corrected for this delay. The presence of radioactivity in the fractions was determined using a Beckman LS 7500 liquid scintillation counter.

Identification of Tritiated Reoxidation Products. The product of reoxidation of (5*R*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ was identified as 8-OH-5-deazaFAD by showing that an aliquot of the filtrate from the reoxidation with CH_2-H_4folate could be rereduced on treatment with $NaBH_4$. Following reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with $NADP^+$, the site of incorporation of tritium in NADPH was shown to be the 4*S* position by repeating the reoxidation under aerobic conditions without adding any NADPH before the reoxidation. Under aerobic conditions, residual native enzyme transfers hydrogen stereospecifically from the 4 pro-*S* position of NADPH to the FAD, and hydrogen bound to the reduced FAD exchanges rapidly with solvent. The reduced native enzyme is rapidly reoxidized by oxygen under aerobic conditions, resulting in stereospecific exchange of tritium from the 4*S* position of NADPH into solvent.

Tritium was identified in the methyl group of CH_3-H_4folate derived from the reoxidation of (5*S*)-[5-^{3}H]-8-OH-5-deazaFADH$_2$ with CH_2-H_4folate by an enzyme-catalyzed transfer of the methyl group to homocysteine to form methionine. Homocysteine, *S*-adenosylmethionine, hydroxycobalamin, and cobalamin-dependent methionine synthase from *Escherichia coli* were incubated for 5 min at 37 °C in 100 mM potassium phosphate buffer, pH 7.2, containing 25 mM dithiothreitol, then mixed with the anaerobically reoxidized methylenetetrahydrofolate reductase mixture, and incubated another 15 min at 37 °C before HPLC analysis. The final concentrations of the methionine synthase substrates in the 100 μL incubation were as follows: 5 mM homocysteine, 190 μM S-adenosylmethionine, and 50 μM hydroxycobalamin. Methionine elutes from a 5 μ Ultrasphere ODS column at 5.5 min in 0.1 M sodium acetate, pH 3.55.[30]

Measurement of Oxidoreductase Activities. Where indicated, the oxidoreductase assays were supplemented with 2 mM FAD or 8-OH-5-deazaFAD. The NADPH-menadione oxidoreductase activity was measured as previously described by Matthews[18] except the total volume was 450 μL and a quartz microcuvette was used. The assay mixture was preincubated for 5 min at 25 °C before initiation of the reaction with menadione. The NADPH-CH_2-H_4folate oxidoreductase assay was measured by the method of Matthews and Haywood[3] with slight modifications. The assay buffer (50 mM potassium phosphate, pH 7.2, 0.3 mM in EDTA and 50 mM in β-mercaptoethanol), NADPH, and flavin (when appropriate) were mixed in the microcuvette, bubbled with argon for 3 min, and then covered with parafilm. Enzyme was added and incubated for 5 min at 25 °C before the addition of CH_2-H_4folate to give a 100 μM final concentration in 410 μL. The NADPH-quinoid-H_2folate oxidoreductase assay was modified from Matthews and Kaufman.[13] A mixture of 300 μL of 50 mM potassium phosphate buffer, containing 0.3 mM EDTA, 0.05% hydrogen peroxide, 2.6 μg of horseradish peroxidase, 166 μM NADPH, and 121 μM H_4folate was incubated for 1 min at room temperature. The absorbance at 340 nm was monitored for 2 min, followed by the addition of 100 μL of a preincubated mixture of buffer plus enzyme, supplemented with flavin where indicated. All reported rates for the NADPH-quinoid-H_2folate oxidoreductase assay have been corrected for the rate of the nonenzymatic reaction. Concentrations of the apo- and holoenzyme stock solutions were determined using Pierce Micro BCA Protein Assay and used to convert activities to specific activities.

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