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Studies on the Methylene/Methyl Interconversion Catalyzed by Methylenetetrahydrofolate Reductase from Pig Liver†

Rowena G. Matthews

ABSTRACT: Methylenetetrahydrofolate reductase catalyzes the interconversion of methylenetetrahydrofolate (CH_2-H_4folate) and methyltetrahydrofolate (CH_3-H_4folate). This study examines whether the exchange of methylene protons with solvent is associated with the enzyme-catalyzed reduction of CH_2-H_4folate to CH_3-H_4folate. Two methods have been used to assess this question. ^{13}C nuclear magnetic resonance spectroscopy of [^{13}C]methyltetrahydrofolate formed by enzymatic reduction of [^{13}C]methylenetetrahydrofolate in buffered D_2O indicates that only one deuterium atom from solvent is incorporated into the methyl group. Studies of the partitioning of tritium label in CH_2-H_4folate between tritium incorporation into product and tritium release to solvent suggest that less than 8.5% exchange of the methylene protons is associated with reduction to CH_3-H_4folate in D_2O. If CH_2-H_4folate is reduced in tritiated D_2O, a 2.75-fold discrimination against the formation of tritiated product is seen. This represents a large kinetic isotope effect on the incorporation of solvent hydrogen into the methyl group (k_D/k_T = 2.75, corresponding to k_H/k_D = 10) and is near the maximum limit for a normal primary kinetic isotope effect on hydrogen transfer to carbon. The intrinsic isotope effect on the methylene/methyl conversion is apparently not suppressed by shielding of the proton-mediating group from solvent or by a high reverse commitment to catalysis. The Appendix of this paper contains derivations for equations describing the relationship between intrinsic isotope effects and the maximum observable isotopic discrimination in the formation of product when the incorporation of a solvent proton is mediated by a shielded multiprotic base, and when the discrimination involves a tracer isotope in bulk solvent. For a shielded proton-mediating group with n protons, this equation is

$$\frac{[\text{product}_H]/[\text{product}_D]}{[\text{solvent}_H]/[\text{solvent}_D]} = \frac{1 + (n-1)k_H/k_D}{n}$$

Methylenetetrahydrofolate reductase (EC 1.1.99.15) catalyzes the interconversion of methylene- and methyltetrahydrofolate. In the presence of suitable electron donors or acceptors, the reaction can be run in either direction under identical conditions of pH, temperature, and solvent composition. Examples of such reactions are shown by eq 1 and 2.

$$\text{NADPH} + \text{CH}_2\text{-H}_4\text{folate} \rightarrow \text{NADP}^+ + \text{CH}_3\text{-H}_4\text{folate} \quad (1)$$

$$\text{CH}_3\text{-H}_4\text{folate} + \text{menadione} \rightarrow \text{CH}_2\text{-H}_4\text{folate} + \text{menadiol} \quad (2)$$

The system offers unique opportunities for the study of a reversible methylene/methyl interconversion using isotopically substituted substrates. Because the methyl group is torsiosymmetric, experiments can be performed to measure intramolecular as well as intermolecular discrimination.

The present study addresses three questions: (1) Is exchange of the methylene protons with solvent associated with the reduction of methylene- to methyltetrahydrofolate? (2) Is the hydrogen introduced during the reduction of methylene- to methyltetrahydrofolate in equilibrium with the solvent? (3) Can the intrinsic isotope effect associated with the conversion of the methylene group to a methyl group be measured?

The results indicate that "wash out" of methylene protons does *not* occur during reduction of methylene- to methyltetrahydrofolate, that the hydrogen introduced during reduction *is* in equilibrium with the solvent, and that the lower limit for the intrinsic isotope effect associated with the methylene/methyl conversion is of the expected magnitude for a fully expressed primary isotope effect (k_D/k_T = 2.75, corresponding to k_H/k_D = 10).

Experimental Procedures

[^{14}C]Formaldehyde (10 mCi/mmol) and tritiated water (5 Ci/mL) were obtained from Amersham Searle, and [^3H]-formaldehyde (85 mCi/mmol) was purchased from New England Nuclear. ^2H_2O (99.8% ^2H) was purchased from Stohler Isotope Chemicals, and [^{13}C]formaldehyde (90% ^{13}C) was purchased from Merck. (6*S*)-H_4folate[1] was prepared by reduction of H_2folate (Sigma Chemical Co.) using a 1.2-fold excess of NADPH and dihydrofolate reductase from *Lactobacillus casei* (sp act. 12 IU/mg at pH 5.5; Liu & Dunlap, 1974). (6*R*)-CH_2-H_4folate was formed by condensation of (6*S*)-H_4folate and formaldehyde; after 15 min, the pH of the solution was raised above 8.6 by addition of 1 M ammonium carbonate. The CH_2-H_4folate was purified by chromatography as previously described (Matthews & Baugh, 1980) except that formaldehyde was omitted from the buffers when labeled CH_2-H_4folate was being purified. Pig liver methylenetetrahydrofolate reductase was purified as described by Matthews & Haywood (1979).

Isotopes were counted in a Beckman LS 7500 spectrometer, and quench was determined by measurement of the Compton edge of an external cesium standard. Aquasol-2 scintillation fluid (New England Nuclear) was used. Sufficient counts were collected to ensure no greater than a 1% standard error. Where dual-labeled compounds were being counted, the

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[1] Abbreviations: H_4folate, tetrahydrofolate; H_2folate, 7,8-dihydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; ^1H NMR, proton nuclear magnetic resonance.

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spectrometer was operated in the automatic quench compensation mode, in which the ^3H and ^{14}C windows were shifted to optimize counting efficiency while minimizing spillover. Corrections for ^3H and ^{14}C spillover and efficiency determinations were made by comparison with a series of quenched ^3H and ^{14}C standards. The dpm calculations were assisted by the use of a computer program written by Brian Matthews, which was based on the method for dpm calculation [see Beckman (1980)] described by Beckman Instruments (Beckman, 1976).

Reduction of [^{14}C,^3H]Methylenetetrahydrofolate in D_2O. Incubations were carried out at 25 °C under dry nitrogen and contained 50 mM phosphate buffer (43.5% K_2HPO_4, 56.5% KH_2PO_4), 0.3 mM EDTA, 2 μM FAD, 125 μM NADPH, 100 μM (6*R*)-CH_2-H_4folate, 50 mM dithiothreitol, and methylenetetrahydrofolate reductase in D_2O. Prior to incubation, the phosphate salts, EDTA, FAD, and dithiothreitol were dissolved in D_2O and lyophilized twice. A 10 mM stock of NADPH in 0.02 M unneutralized tris(hydroxymethyl)-aminomethane hydroxide was similarly treated. Methylenetetrahydrofolate reductase was precipitated from aqueous solution with 80% ammonium sulfate and the precipitate dissolved in D_2O and lyophilized. CH_2-H_4folate was adsorbed onto a 1 × 5 cm column of DEAE-cellulose 52 equilibrated with 0.01 M ammonium carbonate, and the column was rinsed with 25 mL of 0.01 M ammonium carbonate in D_2O. CH_2-H_4folate was eluted with 0.5 M NaCl and 0.01 M ammonium carbonate in D_2O, yielding a 4 mM stock solution of substrate. The course of reduction of [^{14}C,^3H]methylenetetrahydrofolate was monitored by measurement of the decrease in absorbance at 340 nm. As the reaction proceeded, 2-mL aliquots were removed and added to 1-mL volumes of boiling dimedone in 1 M acetate buffer, pH 4.5. The quenched aliquots were heated for 5 min at 95 °C and then cooled. The formaldehyde–dimedone complex was extracted into toluene to determine the concentration of residual substrate as the reaction proceeded. Duplicate 0.8-mL portions of the same quenched sample were used to determine release of tritium to solvent, and the radioactivity associated with solvent was separated from radioactive substrate and product by lyophilization and condensation of the solvent in a cold finger. Control lyophilizations were performed with buffered D_2O to which known amounts of ^3H_2O had been added. Control lyophilizations were also performed with 50 nmol of [^{14}C,^3H]methylenetetrahydrofolate or [^{14}C,^3H]methyltetrahydrofolate after quenching as described above. At the completion of reduction of CH_2-H_4folate, the remaining 10 mL of incubation mixture was applied, without quenching, to a 1 × 15 cm column of DEAE-cellulose 52 which had previously been equilibrated with 0.05 M ammonium acetate, pH 7.2, 20 mM in 2-mercaptoethanol. The column was eluted with a 200-mL linear gradient of 0–1 M NaCl in the equilibrating buffer. Fractions containing CH_3-H_4folate were identified by UV spectroscopy, and the concentration of CH_3-H_4folate was calculated by using an ϵ_{292} of 31 700 M^{-1} cm^{-1} (Blakley, 1969). Aliquots of each fraction were also counted to determine the specific radioactivity of the CH_3-H_4folate.

Incorporation of Solvent Hydrogen during Reduction of CH_2-H_4folate. Incubations were performed at 25 °C under the conditions described in the preceding section, except that unlabeled CH_2-H_4folate was present, and the D_2O solvent contained 0.2% (by volume) ^3H_2O. The sequence of addition of reagents was NADPH, ^3H_2O, CH_2-H_4folate, and finally enzyme. During the course of the reaction, four 100-μL aliquots were removed and diluted into 100-mL volumetric flasks. Duplicate 100-μL portions from each volumetric flask were counted to determine the specific radioactivity of solvent in the incubation mixture. After 1 h, the incubation mixture was applied to a 1 × 15 cm column of DEAE-cellulose 52, previously equilibrated with 0.05 M ammonium acetate, pH 7.2, 20 mM in 2-mercaptoethanol, and the column was washed with 100 mL of the equilibrating buffer. Elution of CH_3-H_4folate was effected as described above. The pooled fractions of CH_3-H_4folate, representing 52% of the total product formed, were diluted 3 ×, applied to a second column of DEAE-cellulose 52, and eluted as before. The specific radioactivity of each fraction was determined as described above.

NMR Spectroscopy of ^{13}C-Enriched CH_3-H_4folate. [^{13}C]Methyltetrahydrofolate was prepared by reduction of [^{13}C]methylenetetrahydrofolate in buffered D_2O or H_2O under the conditions used for reduction of double-labeled CH_2-H_4-folate as described above. [^{13}C]Methylenetetrahydrofolate was formed from a 5:1 molar ratio of [^{13}C]formaldehyde (90% enriched) and (6-*RS*)-H_4folate. After completion of the reduction, the mixture was chromatographed on a 1.5 × 29 cm column of DEAE-cellulose 52 previously equilibrated with 0.05 M ammonium acetate, pH 7.2. The CH_3-H_4folate was eluted with a 600-mL linear gradient of 0.05–1.0 M ammonium acetate, 20 mM in 2-mercaptoethanol. Fractions containing CH_3-H_4folate were pooled and lyophilized twice to remove buffer and mercaptoethanol and then dissolved in 0.1 N NaOH in 50:50 D_2O:H_2O. The final CH_3-H_4folate concentration was 5–9 mM. ^{13}C NMR spectra were recorded at 90 MHz on a Bruker WM-360 spectrometer. The nitrogen flowing over the NMR tube was cooled to 20 °C. A 30° pulse width was used, with an acquisition time of 0.82 s and a delay time of 0.5 s. The chemical shifts are reported relative to an external standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate in D_2O, run under the same conditions.

Results

Reduction of CH_2-H_4folate to CH_3-H_4folate Is Not Associated with Exchange of the Methylene Protons with Solvent. Our initial studies (Matthews & Haywood, 1979) had shown that the hydrogen required for reduction of CH_2-H_4folate to CH_3-H_4folate came from solvent, in agreement with earlier work of Kisliuk (1963) on methylenetetrahydrofolate reductase from *Escherichia coli*. Our studies also suggested that partial exchange of the methylene protons was associated with the enzyme-catalyzed reduction. These conclusions were based on NMR measurements of the proton content of the methyl group of CH_3-H_4folate following reduction in D_2O. However, line broadening of the methyl resonance due to the incorporation of one deuterium atom could lead to erroneously low values for the measured proton content. For this reason, it seemed important to look for exchange of the methylene protons during reduction by alternate methods.

If ^{13}C-enriched [^{13}C]methylenetetrahydrofolate is prepared and then reduced enzymatically in buffered D_2O, a proton-decoupled ^{13}C NMR spectrum of the product can be measured. The presence of deuterium in the methyl group will lead to splitting of the [^{13}C]methyl resonance—a 1:1:1 triplet will be seen if only one deuterium atom is present, but more deuterium atoms will result in the appearance of quintets or septets. Figure 1 shows the ^{13}C NMR spectrum obtained in such an experiment. The 1:1:1 triplet at δ 44.7 has the chemical shift and coupling (20.75 Hz) expected for an *N*-methyl group containing a single deuterium atom. No indication of quintets or septets is seen. Thus, the majority of methyl groups formed during enzymatic reduction of CH_2-H_4folate in D_2O appear to contain only one deuterium atom.

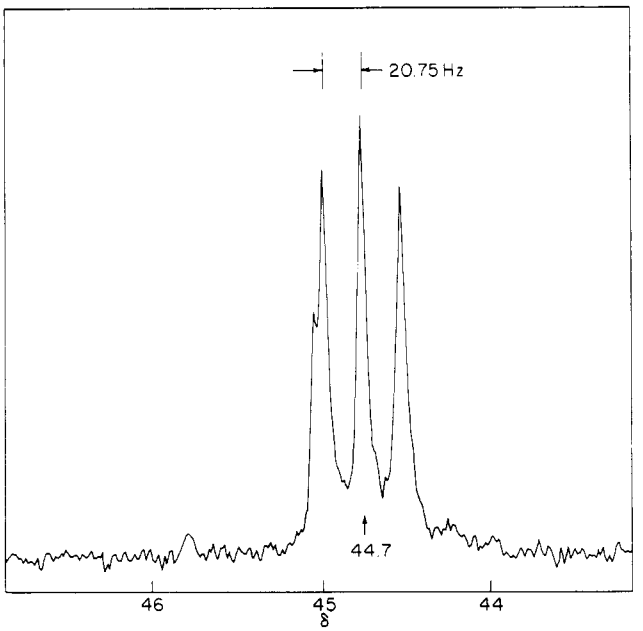


FIGURE 1: Proton-decoupled ^{13}C NMR spectrum of CH_3-H_4folate formed by reduction of [^{13}C]methylenetetrahydrofolate in buffered D_2O. The CH_3-H_4folate was purified prior to spectroscopy as described under Experimental Procedures and then dissolved in 0.1 N NaOH in 1:1 D_2O:H_2O.

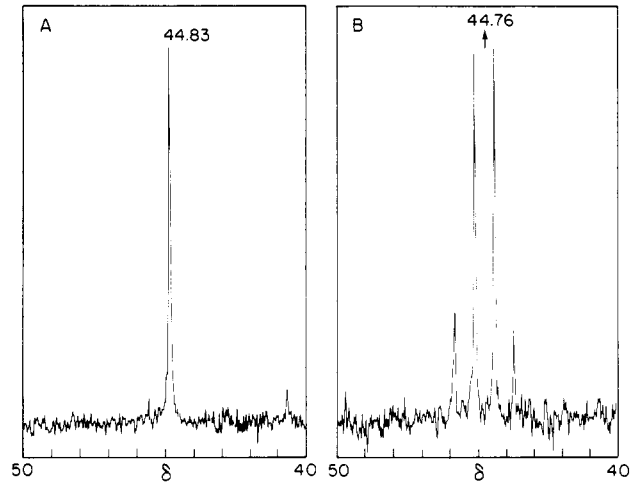


FIGURE 2: ^{13}C NMR spectra of CH_3-H_4folate formed by reduction of [^{13}C]methylenetetrahydrofolate in buffered H_2O. (A) Spectrum taken with proton decoupling. (B) Spectrum taken with off-resonance decoupling.

A control experiment to establish the chemical shift of the fully protonated *N*-methyl group of CH_3-H_4folate is shown in Figure 2. For this experiment, CH_3-H_4folate was prepared by enzymatic reduction of CH_2-H_4folate in buffered H_2O. In general, the chemical shift of a CH_2D group is expected to be 0.1–0.3 ppm toward higher field than that of the corresponding CH_3 group (Levy & Nelson, 1972). As shown in Figure 2A, the methyl group prepared by reduction in H_2O has a chemical shift of 44.8 ppm, and its identity as a methyl group is confirmed by the 1:3:3:1 quartet seen in the off-resonance decoupled spectrum shown in Figure 2B.

A second means of looking for exchange of methylene protons associated with the reduction of CH_2-H_4folate to CH_3-H_4folate involves the measurement of the ratio of tritium released to solvent over tritiated product formed ($[T^+]/[product_T]$) when [^{14}C,^3H]methylenetetrahydrofolate is reduced in buffered D_2O. If significant exchange of methylene protons is associated with reduction, then tritium

Table I: Determination of Partitioning of Tritium Associated with the Reduction of [^{14}C,^3H]Methylenetetrahydrofolate

% conversion	dpm released to solvent per 0.5 mL		dpm of ^3H released per nmol of product	$[T^+]$ / $[product_T]$
	^3H	^{14}C		
0.0[a]	2352	8.2		
7.1	3196	7.2	244	0.0034
21.6	4347	22.5	198	0.0026
33.5	5173	10.6	180	0.0024
45.6	7471	34.8	241	0.0032
60.3	10571	25.4	292	0.0039
0.0[b]	1946	63.0		
37.1	6554	152.8	267	0.0035
41.8	6533	125.9	236	0.0031
58.1	10509	200.0	317	0.0042
64.4	10664	106.9	291	0.0038
CH_3-H_4folate[c]	1929	20.0		

[a] In this experiment, the concentration of CH_2-H_4folate was initially 93.3 μM, and the $^3H/^{14}C$ ratio of the substrate was 20.77. The specific radioactivity of product at the end of the reaction was 75 625 dpm of ^3H/nmol. [b] In this experiment, the concentration of CH_2-H_4folate was initially 93.0 μM, and the $^3H/^{14}C$ ratio of the substrate was 46.89. The specific radioactivity of the product at the end of the reaction was 76 650 dpm of ^3H/nmol. [c] A 0.5-mL aliquot of CH_3-H_4folate, 79.7 μM and 76 650 dpm of ^3H/nmol, was quenched with dimedone and lyophilized. An aliquot of the collected solvent was counted to determine disintegrations per minute released.

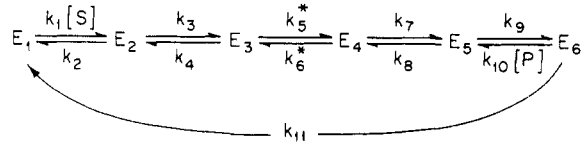
should be released to solvent during reduction. Table I shows the results of such an experiment. The double-labeled CH_3-H_4folate was isolated at the completion of the reaction and purified by chromatography as described under Experimental Procedures, and its specific radioactivity was determined. The specific radioactivity of the initial CH_2-H_4folate cannot be determined directly because of the possibility that the substrate will be contaminated with H_4folate, formed by dissociation of formaldehyde during purification. However, of we assume that the specific radioactivity of ^{14}C will remain unchanged during the reduction of CH_2-H_4folate, determination of the $^3H/^{14}C$ ratio of the initial substrate permits determination of the specific radioactivity for ^3H. The specific radioactivity of the substrate, CH_2-H_4folate, for ^3H was calculated to be 77 600 dpm/nmol, while that for product was 76 650 dpm/nmol. The ratio of tritium released to tritiated product was then 0.0035 and remained constant as the reaction proceeded. Control experiments established that the release of tritium to solvent which was observed did not result from the quenching and lyophilization of either substrate or product and that recovery of added ^3H_2O was quantitative under these lyophilization conditions.

Comparison of the specific radioactivites for ^3H of product vs. initial substrate (76 650 vs. 77 600 dpm/nmol) indicates that about 1% of the initial tritium content is not recovered in the product. However, this value for tritium released per nanomole of product formed is determined as the difference between large numbers and although similar in magnitude to the number obtained by direct measurement of tritium release to solvent (0.35%) will be subject to considerably greater errors.

The $^3H/^{14}C$ ratio of the residual substrate was also determined at various stages of the reaction. There was no detectable intermolecular discrimination against unlabeled substrate.

These experiments qualitatively suggest that the extent of exchange of methylene protons during reduction is very low. A more quantitative analysis of the results requires that we

Scheme I



consider the statistics associated with methylene/methyl interconversion. Scheme I represents a generalized mechanism for the conversion of CH_2-H_4folate (designated S) to CH_3-H_4folate (designated P). As discussed by Northrop (1975), such a scheme can be used to represent one of the half-reactions of a ping-pong mechanism, where k_{11} represents the net rate constant for the other half-reaction. In the case of methylenetetrahydrofolate reductase, the available evidence is fully consistent with a ping-pong mechanism (Matthews & Haywood, 1979; Daubner & Matthews, 1982), and the half-reaction involving reduction of the flavin by NADPH is irreversible (Daubner & Matthews, 1982). The rate constants k_{5*} and k_{6*} have been chosen to represent the isotopically sensitive conversion of the methylene group to a methyl group. No specific mechanism has been assumed; the scheme merely allows for reversible steps preceding and following the methylene/methyl interconversion. The most likely mechanism is shown in Scheme II and involves ring opening of CH_2-H_4folate to form a 5-iminium cation (**3**), tautomerization of the 5-iminium cation to form quinonoid 5-CH_3-H_2folate (**4**) in an isotopically sensitive step, and then reduction of **4** to form CH_3-H_4folate (**5**) (Matthews & Haywood, 1979; Matthews & Kaufman, 1980). If exchange of methylene protons with solvent were to occur during reduction, it would require reversal of the methylene/methyl interconversion prior to the release of product. Such exchange might occur if product release or reduction of quinonoid CH_3-H_2folate were rate limiting in catalysis. If $k_{7'}$ is used to describe a *net* rate constant (Cleland, 1975) for the events occurring after the methylene/methyl interconversion, significant exchange of methylene protons would require that k_{6H} and $k_{7'}$ be of similar magnitude. The dependence of $[T^+]/[product_T]$ on both the ratio of $k_{7'}/k_{6H}$ and the intrinsic isotope effect on k_6 is derived in Scheme III and given by eq 3. The very low value observed

$$[T^+]/[product_T] = \frac{1}{3(k_{7'}/k_{6H})(k_{6H}/k_{6T})} \qquad (3)$$

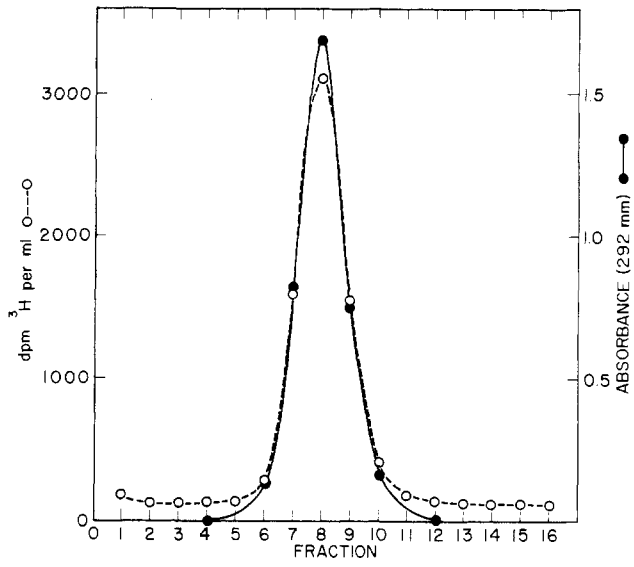
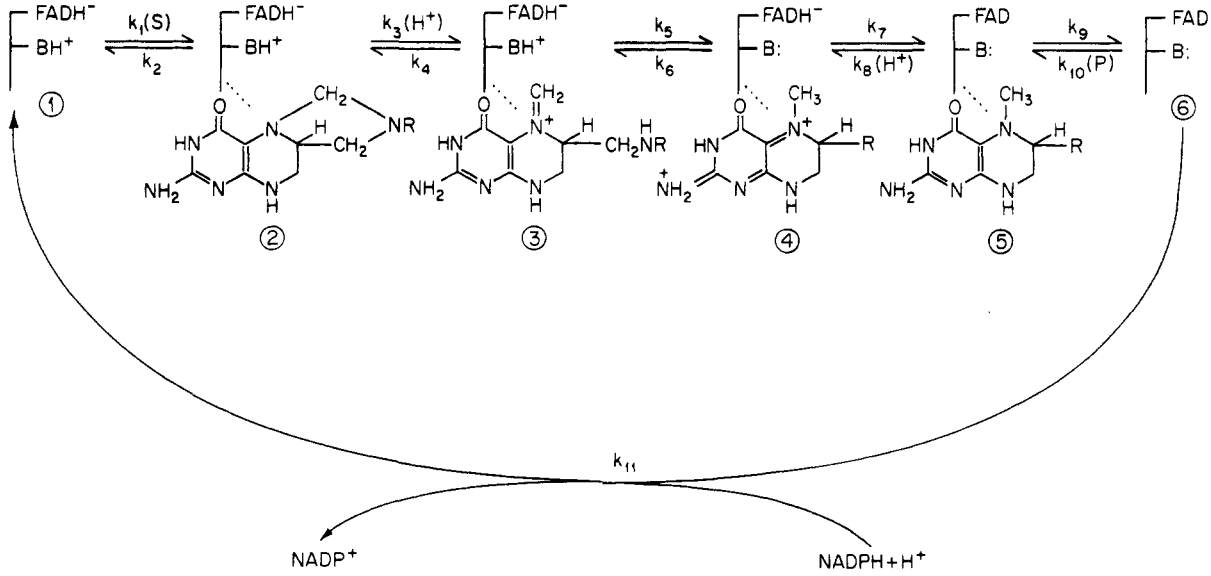


FIGURE 3: Determination of the specific radioactivity (dpm of ^{3}H/nmol) of CH_3-H_4folate formed by reduction of CH_2-H_4folate in tritiated D_2O. The CH_3-H_4folate product was purified by chromatography on DEAE-cellulose 52 as described under Experimental Procedures. This figure shows the elution profile obtained on rechromatography on a second DEAE-cellulose 52 column. The radioactive material was identified as CH_3-H_4folate by UV spectroscopy and by its characterisic position in the elution profile.

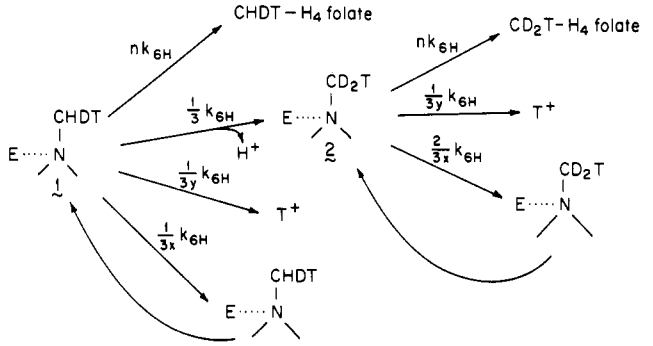
for $[T^+]/[product_T]$ (0.0035) requires that the intrinsic isotope effect on k_6 and/or the ratio $k_{7'}/k_{6H}$ be large. If we assume the maximal normal value for the intrinsic isotope effect on k_6 (k_{6H}/k_{6T} = 30), we can derive an estimate of the *minimum* value for the ratio $k_{7'}/k_{6H}$. This minimum value is 3.7 and would result in a maximum of 8.5% exchange of the methylene protons associated with reduction of CH_2-H_4folate to CH_3-H_4folate in D_2O. Thus, two independent lines of evidence suggest that there is a very low rate of exchange of the methylene protons during reduction.

Hydrogen Introduced during Reduction of CH_2-H_4folate to CH_3-H_4folate Is in Equilibrium with Solvent. The isotopic discrimination associated with the incorporation of solvent into the methyl group during reduction of CH_2-H_4folate has been measured by reducing CH_2-H_4folate in tritiated D_2O of known specific radioactivity and then determining the specific radioactivity of the isolated product, CH_3-H_4folate. The results

Scheme II



Scheme III: Partitioning Scheme for Exchange of Tritium Associated with the Reduction of [^{3}H]Methylenetetrahydrofolate in Buffered D_2O[a]



[a] Loss of H^+ or T^+ to solvent (D_2O) is irreversible, as is product release. In contrast, loss of a D^+ to solvent (by k_6) always results in reincorporation of solvent D^+ (by k_5) and if the reaction is carried to completion will result in no net change in the partitioning. This is indicated by a loop. Let $x = k_{6H}/k_{6D}$, $y = k_{6H}/k_{6T}$, and $n = k_{7'}/k_{6H}$.

of such an experiment are shown in Figure 3. When CH_2-H_4folate was reduced in tritiated D_2O (157.4 dpm/nmol), the isolated product had a specific radioactivity of 57.2 dpm/nmol. [Although the isolation of CH_3-H_4folate was performed without first denaturing the protein, which might result in enzyme-catalyzed loss of tritium from product during chromatography, two lines of evidence suggest that such loss of tritium does not in fact occur during the isolation procedure. Addition of fully protonated CH_3-H_4folate to an incubation mixture in D_2O followed by isolation of the CH_3-H_4folate by chromatography results in a product containing three protium atoms per methyl group as judged by ^{1}H NMR spectroscopy. Also, as discussed above, reduction of [^{14}C,^{3}H]methylenetetrahydrofolate in D_2O followed by isolation of the product under exactly the same conditions as were followed in this "wash in" experiment results in a product which retains 99% of the tritium initially present in the substrate, again indicating that the isolation procedure does not result in tritium release from product.] The incorporation of solvent tritium into the product is expected if the hydrogen required for reduction comes from the solvent. Discrimination against incorporation of solvent tritium is also expected due to the intrinsic isotope effect k_{5D}/k_{5T}. However, as shown in eq 4, the intermolecular

$$\frac{(V/K)_D}{(V/K)_T} = \frac{k_{5D}/k_{5T} + C_{f'} + C_r}{1 + C_{f'} + C_r} \quad (4)$$

discrimination against incorporation of tritium from solvent may be suppressed by a high reverse commitment to catalysis, $C_r = k_6/k_{7'}$ (Northrop, 1981), or by shielding of the hydrogen donor from bulk solvent (represented by $C_{f'}$, the forward commitment to catalysis of the *proton* as distinct from the substrate).

As described above, a 2.75-fold discrimination against tritiated product has been observed. Since the ^{13}C NMR studies establish that *one* solvent hydrogen is incorporated into the product methyl group, a 2.75-fold discrimination corresponds to an apparent D/T isotope effect of 2.75 or to an H/D isotope effect of 10 if the Swain–Schaad relationship (Swain et al., 1958) holds. This represents the upper limit for a normal primary kinetic isotope effect on a proton transfer to carbon (Melander & Saunders, 1980) and suggests that suppression of the intrinsic isotope effect does *not* occur under these conditions. Hence, the reverse commitment (C_r) must be low, which in turn requires that $k_{7'}$ be greater than k_6, in agreement with the requirement inferred from determination of $[T^+]/[\text{product}_T]$.

Since this is an enzymatic reduction, the immediate proton donor may be a bound water or an acidic group on the protein itself. If the acidic group were monoprotic (e.g., an imidazolium residue), the observation of pronounced isotopic discrimination against formation of tritiated product would serve to indicate that the proton-mediating group was not shielded from bulk solvent during catalysis. However, if the proton-mediating group were diprotic or triprotic, intramolecular discrimination against formation of tritiated product could occur, even if the residue were shielded from solvent during catalysis. Northrop (1981) has discussed the limits on the expression of solvent isotope effects for multiprotic residues which are shielded from bulk solvent. He derived equations for the maximal isotopic discrimination which could be observed with shielded multiprotic acid/base catalysts in a 50:50 mixture of H_2O and D_2O. Equations analogous to his are derived in the Appendix for discrimination against tracer isotopes in bulk solvent. For a diprotic acid, the analogous equation is shown in eq 5, while eq 6 describes the triprotic acid. For the experiment described above, the bulk solvent

$$\frac{[\text{product}_H]/[\text{product}_D]}{[\text{solvent}_H]/[\text{solvent}_D]} = \frac{1 + k_H/k_D}{2} \quad (5)$$

$$\frac{[\text{product}_H]/[\text{product}_D]}{[\text{solvent}_H]/[\text{solvent}_D]} = \frac{1 + 2k_H/k_D}{3} \quad (6)$$

was D_2O rather than H_2O, and the tracer isotope was tritium rather than deuterium. If we assume a normal intrinsic isotope effect ($k_D/k_T \leq 2.75$, corresponding to $k_H/k_D \leq 10$), then eq 5 and 6 require that the observed isotopic discrimination against tritiated product in bulk D_2O be less than 2.3-fold (corresponding to an observed discrimination against deuterated product in bulk H_2O of less than 7-fold). Since the measured isotopic discrimination against tritiated product in bulk D_2O was 2.75-fold, this observation is inconsistent with mechanisms involving a di- or triprotic shielded base.

Discussion

The present results establish that reduction of CH_2-H_4folate to CH_3-H_4folate, as catalyzed by methylenetetrahydrofolate reductase from pig liver, is *not* associated with significant exchange of the methylene protons with solvent. These observations have important implications for the stereochemical analysis of this reaction. Had significant exchange of the methylene protons been associated with reduction, then reduction of stereospecifically labeled [^{3}H]methylenetetrahydrofolate in D_2O would have resulted also in the production of achiral CD_2T-H_4folate due to the exchange reaction, and this could have been interpreted as a loss of chirality associated with the reduction, even if the enzyme-catalyzed reduction were in fact completely stereospecific. In the absence of significant methylene proton exchange, stereochemical analysis of the enzyme-catalyzed reduction should be straightforward.

These results do not permit distinction between the mechanistic pathway shown in Scheme II and the alternate pathway involving direct reduction of the 5-iminium cation by a hydride equivalent. However, our earlier observation that the enzyme catalyzes the NADPH-linked reduction of quinonoid forms of dihydropterins and dihydrofolate favors the pathway shown in Scheme II (Matthews & Kaufman, 1980). If this sequence correctly describes the reaction, the observation of pronounced isotopic discrimination in the incorporation of solvent protons would require that intermediate **3** of Scheme II have a lifetime long enough to permit solvent equilibration. (Note that **3** is

a canonical form of the carbanion which would be formed by removal of a proton from the methyl group of **4**.) Jencks (1980) has discussed the suppression of discrimination between hydrogen isotopes in product formed by protonation of highly unstable carbanions in solvents of mixed isotopic composition. In contrast, resonance-stabilized carbanions with sufficiently low pK values have lifetimes long enough to permit isotopic discrimination between proton donors. Oxidation of the tetrahydropterin ring of **5** to the highly electron-deficient quinonoid dihydropterin ring of **4** should facilitate removal of a proton from the N^5-methyl group, with the quinonoid dihydropterin acting as an electron sink for the electrons of the carbanion in much the same way that pyridoxal phosphate stabilizes carbanions formed by removal of the α hydrogen from amino acids. The enzyme-bound Schiff base between glycine and pyridoxal phosphate formed by mitochondrial serine hydroxymethyltransferase appears to have a pK of about 9.3 for dissociation of the α hydrogen of the glycyl residue (Schirch & Peterson, 1980), and the pK of the carbanion formed by removal of a proton from the methyl group of **4** could be in the same range.

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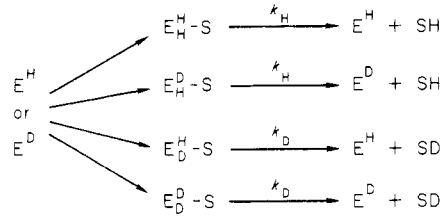
Appendix

Derivation of Equations Describing Solvent Isotope Effects Where the Proton Mediators Are Multiprotic and Shielded and Discrimination Involves a Tracer Isotope in Bulk Solvent. Let us first consider the situation where the proton mediator is diprotic, and the solvent is H_2O containing tracer deuterium.



Since deuterium is only present in tracer concentrations, we will neglect the contribution of enzyme in which the diprotic acid contains two deuterium atoms because this species is statistically unlikely to be formed. Following the arguments outlined by Northrop (1981), we define pathway turnover rates:

$$r_1 = k_H[E_H^H\text{–S}] \qquad r_2 = k_H[E_H^D\text{–S}] \qquad r_3 = k_D[E_D^H\text{–S}]$$

Both r_1 and r_2 lead to the formation of SH, but since the concentration of deuterium is much lower than that of protium, the concentration of E_H^D–S will be much less than that of E_H^H–S, and thus the contribution of r_2 to the formation of SH will be neglected. If we assume that the diprotic acid is shielded from bulk solvent after substrate is bound to the enzyme, but that positioning of the hydrogen atoms of the acid is in rapid equilibrium relative to the rate of transfer of hydrogen to substrate, then E_H^D–S and E_D^H–S will always be present in equal concentrations. The pathway turnover through r_2 and r_3 can now be written

$$r_2 + r_3 = (k_H + k_D)[E_H^D\text{–S}] = \frac{k_H + k_D}{2}([E_H^D\text{–S}] + [E_D^H\text{–S}])$$

Given the solvent composition $[\text{solvent}_D]/[\text{solvent}_H]$, we can now calculate the relationship between r_1 and $r_2 + r_3$.

$$r_2 + r_3 = 2r_1[\text{solvent}_D]/[\text{solvent}_H]$$

Substituting, we obtain

$$\frac{k_H + k_D}{2}([E_H^H\text{–S}] + [E_D^D\text{–S}]) = 2k_H[E_H^H\text{–S}][\text{solvent}_D]/[\text{solvent}_H]$$

Rearranging

$$\frac{[E_H^H\text{–S}]}{[E_H^D\text{–S}] + [E_D^H\text{–S}]} = \frac{1 + k_H/k_D}{4k_H/k_D}[\text{solvent}_H]/[\text{solvent}_D]$$

Now the total concentration of enzyme species, E_T, is given by

$$E_T = [E_H^H\text{–S}] + [E_D^H\text{–S}] + [E_H^D\text{–S}] = \{[E_D^H\text{–S}] + [E_H^D\text{–S}]\}\left\{1 + \frac{1 + k_H/k_D}{4k_H/k_D}\frac{[\text{solvent}_H]}{[\text{solvent}_D]}\right\}$$

From these, we can calculate

$$\frac{r_1}{E_T} = \frac{k_H\dfrac{[\text{solvent}_H]}{[\text{solvent}_D]}\left(\dfrac{1 + k_H/k_D}{4k_H/k_D}\right)}{1 + \left(\dfrac{1 + k_H/k_D}{4k_H/k_D}\right)\left(\dfrac{[\text{solvent}_H]}{[\text{solvent}_D]}\right)}$$

$$\frac{r_3}{E_T} = \frac{k_D/2}{1 + \left(\dfrac{1 + k_H/k_D}{4k_H/k_D}\right)\left(\dfrac{[\text{solvent}_H]}{[\text{solvent}_D]}\right)}$$

$$\frac{[\text{product}_H]/[\text{product}_D]}{[\text{solvent}_H]/[\text{solvent}_D]} = \frac{r_1/r_3}{[\text{solvent}_H]/[\text{solvent}_D]} = \frac{1 + k_H/k_D}{2}$$

Following a similar derivation for a shielded n-protic mechanism yields

$$\frac{[\text{product}_H]/[\text{product}_D]}{[\text{solvent}_H]/[\text{solvent}_D]} = \frac{1 + (n - 1)k_H/k_D}{n}$$

The peculiar form of these equations may be explained as follows: Compensatory distribution leads to complete suppression of the isotope effect associated with a monoprotic base. If the base is diprotic and rapidly exchanges D for H at the transfer site, compensatory distribution raises the concentration of $([E_D^H\text{–S}] + [E_H^D\text{–S}])$ by an amount equal to k_H/k_D, and the compensatory increase in $[E_D^H\text{–S}]$ is only enough to suppress half the intrinsic isotope effect. In the triprotic case, the compensatory increase in $[E_{HHD}\text{–S}]$ is equal to $k_H/(3k_D)$ and thus can only suppress one-third of the intrinsic isotope effect.

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Inactivation of Rat Liver *S*-Adenosylhomocysteinase by Iodoacetamide†

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ABSTRACT: *S*-Adenosylhomocysteinase (EC 3.3.1.1) from rat liver is inactivated by iodoacetamide following pseudo-first-order reaction kinetics. The apparent first-order rate constant for inactivation is proportional to the concentration of the modifier, and a value of 7.55 M^{-1} min^{-1} is obtained for the second-order rate constant at pH 9.06 and 25 °C. Amino acid analysis of the modified enzyme shows the formation of *S*-(carboxymethyl)cysteine. No peaks corresponding to N^{ϵ}-(carboxymethyl)- and N^{ϵ},N^{ϵ}-bis(carboxymethyl)lysines, *N*-(carboxymethyl)histidines, *S*-(carboxymethyl)homocysteine, homoserine, and homoserine lactone are detected. Glycolic acid is also not found in the acid hydrolysate of the modified enzyme, indicating the absence of modification at carboxyl residues. These results and the finding that the number of residues modified as determined by the incorporation of iodo[1-^{14}C]acetamide is equal to the number of cysteine residues lost by modification establish the site of modification as cysteine residues. Kinetics of inactivation and incorporation of the label from iodo[1-^{14}C]acetamide show that two among three modifiable residues per enzyme subunit are essential for activity and the modification of either results in complete inactivation. The inactivation by iodoacetamide does not involve alteration in the molecular size of enzyme nor release of the bound NAD^+. The modified enzyme still retains the capacity to bind adenosine and to oxidize it as evidenced by the reduction of enzyme-bound NAD^+ but does not catalyze the exchange of the 4′ proton with solvent. Thus, it is suggested that the inability of the modified enzyme to catalyze the overall reaction is due to the failure to abstract the 4′ proton in the catalytic cycle.

S-Adenosylhomocysteinase (EC 3.3.1.1), which catalyzes the cleavage and synthesis of the thioether bond of *S*-adenosyl-L-homocysteine, has been purified to homogeneity from a variety of sources (Ueland & Døskeland, 1977; Guranowski & Pawełkiewicz, 1977; Richards et al., 1978; Palmer & Abeles, 1979; Schatz et al., 1979; Chabannes et al., 1979; Kajander & Raina, 1981; Fujioka & Takata, 1981). The enzyme isolated from rat liver is a tetramer consisting of apparently identical subunits with M_r 47 000 (Fujioka & Takata, 1981). Like *S*-adenosylhomocysteinases from beef liver (Richards et al., 1978; Palmer & Abeles, 1976, 1979) and human placenta (Hershfield et al., 1979), the rat liver enzyme contains 1 mol of tightly bound NAD^+/mol of subunit, which is essential for activity.

The pathway of *S*-adenosylhomocysteinase-catalyzed reaction has been studied in detail by Palmer & Abeles (1979), who showed that *S*-adenosylhomocysteine and adenosine are first oxidized to 3′-keto derivatives by the enzyme-bound NAD^+,[1] followed by a series of reactions to yield 3′-keto-4′,5′-dehydroadenosine. Addition of water or L-homocysteine to this central intermediate and subsequent reactions that are the reversal of the reaction paths result in the formation of product. Although the reaction pathway is fairly well understood, little is known about the active site residues that participate in catalysis or binding of substrates. The only type of residue that appears to be essential for activity is cysteine. *S*-Adenosylhomocysteinases from yellow lupin seeds (Guranowski & Pawełkiewicz, 1977) and rat brain (Schatz et al., 1979) are shown to be inhibited by *p*-(chloromercuri)benzoate and *N*-ethylmaleimide. Preliminary studies in this laboratory have shown that the rat liver enzyme is also inhibited by a variety of sulfhydryl reagents including *p*-(chloromercuri)-benzoate, 5,5′-dithiobis(2-nitrobenzoate), iodoacetate, iodoacetamide, 4-(iodoacetamido)salicylate, and *N*-ethylmaleimide. Complete inhibition of the enzyme activity could be achieved by each of these reagents. The present paper describes the results of chemical modification studies with iodoacetamide and shows that the rat liver *S*-adenosylhomocysteinase contains

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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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