Binding of (6*R,S*)-Methyltetrahydrofolate to Methyltransferase from *Clostridium thermoaceticum*: Role of Protonation of Methyltetrahydrofolate in the Mechanism of Methyl Transfer†

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ABSTRACT: The methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase (MeTr) from *Clostridium thermoacetium* catalyzes transfer of the N^5-methyl group of (6*S*)-methyltetrahydrofolate (CH_3-H_4folate) to the cob(I)amide center of a corrinoid/iron−sulfur protein (CFeSP), forming H_4folate and methylcob(III)amide. We have investigated binding of ^{13}C-enriched (6*R,S*)-CH_3-H_4folate and (6*R*)-CH_3-H_4folate to MeTr by ^{13}C NMR, equilibrium dialysis, fluorescence quenching, and proton uptake experiments. The results described here and in the accompanying paper [Seravalli, J., Shoemaker, R. K., Sudbeck, M. J., and Ragsdale, S. W. (1999) *Biochemistry 38*, 5728−5735] constitute the first evidence for protonation of the pterin ring of CH_3-H_4folate. The pH dependence of the chemical shift in the ^{13}C NMR spectrum for the N^5-methyl resonance indicates that MeTr decreases the acidity of the N^5 tertiary amine of CH_3-H_4folate by 1 p*K* unit in both water and deuterium oxide. Binding of (6*R,S*)-CH_3H_4folate is accompanied by the uptake of one proton. These results are consistent with a mechanism of activation of CH_3-H_4folate by protonation to make the methyl group more electrophilic and the product H_4folate a better leaving group toward nucleophilic attack by cob(I)amide. When MeTr is present in excess over (6*R,S*)-$^{13}CH_3$-H_4folate, the ^{13}C NMR signal is split into two broad signals that reflect the bound states of the two diastereomers. This unexpected ability of MeTr to bind both isomers was confirmed by the observation of MeTr-bound (6*R*)-$^{13}CH_3$-H_4folate by NMR and by the measurement of similar dissociation constants for (6*R*)- and (6*S*)-CH_3-H_4folate diastereomers by fluorescence quenching experiments. The transversal relaxation time (T_2) of $^{13}CH_3$-H_4folate bound to MeTr is pH independent between pH 5.50 and 7.0, indicating that neither changes in the protonation state of bound CH_3-H_4folate nor the previously observed pH-dependent MeTr conformational change contribute to broadening of the ^{13}C resonance signal. The dissociation constant for (6*R,S*)-CH_3-H_4folate is also pH independent, indicating that the role of the pH-dependent conformational change is to stabilize the transition state for methyl transfer, and not to favor the binding of CH_3-H_4folate.

The methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase (MeTr)[1] from *Clostridium thermoaceticum* catalyzes the reversible methyl transfer from the N^5-methyl group of (6*S*)-CH_3-H_4folate to the cob(I)amide center of a corrinoid iron−sulfur protein (CFeSP) or to free cob(I)alamin (eq 1). This reaction is a key step in the Wood-Ljungdahl (*1*−*3*) pathway of acetyl-CoA synthesis. The methyl group is then transferred from cob(III)amide to CO dehydrogenase/acetyl-CoA synthase (CODH/ACS), where it combines with CoA and a carbonyl group derived from CO to generate acetyl-CoA.

$$CH_3\text{-}H_4\text{folate} + \text{cob(I)amide} \xrightleftharpoons{\text{MeTr}} H_4\text{folate} + CH_3\text{-cob(III)amide} \quad (1)$$

MeTr is a homodimeric enzyme of identical 28 kDa subunits (*4*). The MeTr gene has been cloned, sequenced, and actively overexpressed in *Escherichia coli* (*5*, *6*). MeTr has also been crystallized (*7*). The physiological acceptor of the methyl group of CH_3-H_4folate is the corrinoid/iron−sulfur protein (CFeSP). This protein is composed of two subunits with molecular masses of 33 and 55 kDa (*8*, *9*) The 33 kDa subunit contains the corrinoid cofactor, 5′-methoxybenzimidazolylcobamide; whereas a low-potential $[Fe_4S_4]^{2+/1+}$ cluster is located in the 55 kDa subunit (*9*, *10*). The cluster is involved in reductive activation of the protein to maintain the cobalt center in the cob(I)amide state (*11*). Stopped-flow studies have shown that the methyl transfer reaction occurs through a nucleophilic attack by the cob(I)amide state of the CFeSP on the methyl group of (6*S*)-CH_3-H_4folate (*12*). Thus, during catalysis, the CFeSP cycles between the cob(I)amide

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[1] Abbreviations: H_4folate, tetrahydrofolate; CH_3-B_{12}, methylcobalamin; CH_3-H_4folate, methyltetrahydrofolate; CFeSP, corrinoid/iron−sulfur protein; MeTr, methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; SHE, standard hydrogen electrode; MES, 2-(*N*-morpholino)-ethanesulfonate; DEAE cellulose, diethylaminoethane cellulose; IPTG, isopropylthiogalactoside; bis-ANS, 4,4′-bis-1-phenylamino-8-naphthalenesulfonate.

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and the methylcob(III)amide states. Control of cobalt coordination chemistry by removing the lower axial cobalt ligand and imposing other subtle electronic changes on the cobalt center facilitates reductive activation and enhances its methyl transfer ability (*9, 13*−*15*).

Under steady-state conditions, the MeTr reaction is strongly pH dependent with a pK_a of 5.1 in the forward (methylation of CFeSP) and reverse (methylation of H_4folate) directions (*12*). These findings led to the conclusion that an ionization in MeTr, not in the substrates, was primarily responsible for the pH dependence. Studies with model compounds for CH_3-H_4folate indicate that protonation at N^5 or coordination to an electrophilic center can activate CH_3-H_4folate (*16, 17*). Such activation would render the methyl group more electrophilic and H_4folate a better leaving group. Activation of CH_3-H_4folate by protonation has been considered to contribute to catalysis by MeTr; however, this does not appear to be a rate-limiting step in the reaction (*12, 18*). Studies of the pH dependence of tryptophan intrinsic fluorescence in MeTr (pK_a of 5.10) led to the proposal that a hydrophobic region of the protein containing at least one tryptophan residue becomes more solvent exposed at lower pH, which facilitates binding of CH_3-H_4folate and H_4folate (*18*). Using the extrinsic fluorescence probe 4,4′-bis-1-phenylamino-8-naphthalene sulfonate (bis-ANS) to monitor the exposure of hydrophobic residues to solvent, a pH-dependent conformational change in MeTr was observed that is fast enough (40 s^{-1}) to be kinetically competent for the transmethylation reaction. Furthermore, binding of bis-ANS to MeTr is strongly pH dependent, and it is an inhibitor of the methyl transfer reaction. These observations indicate (*18*) that CH_3-H_4folate binding itself may be pH dependent. However, direct measurement of the pH dependence of CH_3-H_4folate binding is difficult because MeTr is unstable at pH values below its isoelectric point of 4.80. Since the pK_a of CH_3-H_4folate is 4.82 (*19*), data acquisition is limited to a pH region where the predominant species of CH_3-H_4folate is the inactive unprotonated form.

Knowles has pointed out that studying the pH dependence of kinetic parameters is meaningful only if there is one ionization state of the active site that catalyzes conversion of substrate to product, if there are no parallel pathways during the reaction over the entire pH range, and if the elementary step affected by the ionization is rate-determining over the entire pH range (*20*). That there is a pH-dependent conformational change in the protein that matches the kinetic pK_a adds ambiguity to any interpretation of pH-dependent kinetics of MeTr. In summary, it is difficult to extract the ionization state of bound CH_3-H_4folate from kinetic studies. The best way to obtain reliable information on the ionization of a substrate or an enzyme active site is to directly observe the titrating group. In the present work, we address this question using nuclear magnetic resonance (NMR) spectroscopy to examine the binding of ^{13}C-enriched $^{13}CH_3$-H_4folate to MeTr. The pK_a of the bound substrate increases by a full pH unit in both H_2O and D_2O relative to the value in solution. This result implies that, at equilibrium, MeTr binds the protonated form of the substrate 10-fold stronger than the unprotonated form. Titrations of MeTr with (6*R,S*)-CH_3-H_4folate show that the increase in basicity of (6*R,S*)-CH_3-H_4folate upon binding is manifested by the uptake of a proton. The dissociation constant for CH_3-H_4folate is, nevertheless, pH independent. A surprising conclusion from these studies is that MeTr interacts with both (6*S*) and (6*R*) diastereomers of CH_3-H_4folate.

MATERIALS AND METHODS

Materials. (6*R,S*)-H_4folate, MES free acid and sodium salt, DEAE cellulose, and cellulose were purchased from Sigma. (6*R,S*)-CH_3-H_4folate for proton uptake studies was from Schirck's Laboratories (Jona, Switzerland), (6*R*)-H_4folate was a generous gift from Dr. Rudolf Moser at EPROVA AG (Switzerland), (6*R,S*)- $^{14}CH_3$-H_4folate was purchased from Amersham Pharmacia Biotech, and (6*S*)-CH_3-H_4folate was a gift from SAPEC SA (Lugano, Switzerland). Deuterium oxide was purchased from Isotec; 99.9% labeled [^{13}C]-formaldehyde was purchased from Cambridge Isotopes. Acetone and $Ti^{III}Cl_3$ were purchased from Aldrich. IPTG was obtained from GibcoBRL. Phenyl Sepharose (6 Fast Flow low sub) was from Pharmacia-Biotech. All other chemicals are from either Sigma or Aldrich and were used without further purification.

Substrate Synthesis. (6*R,S*)-CH_3-H_4folate enriched with ^{13}C at the N^5-methyl group was prepared by reductive alkylation of H_4folate with [^{13}C]formaldehyde and $NaBH_4$ by a modification of the method of Gupta and Huennekens (*21*). The reaction was carried out at 18 °C for 3 h, instead of at 37 °C for 1 h, and at a pH of 8.0 instead of 7.50, to avoid the formation of N^{10}-CH_3-H_4folate, as has previously been reported (*22*). The synthesis and purification were done in an anaerobic glovebox from Coy Laboratories (Ann Arbor, MI). Tetrahydrofolic acid (500 mg, 70% pure, 0.65 mmol) was dissolved, with addition of 1 M sodium hydroxide, into 11 mL of 0.5 M potassium phosphate (KP_i) buffer, pH 8.0, containing 1 mL of 20% H^{13}CHO (6.45 mmol) and 1 mL of 20 mM mercaptoethanol. After cooling to 4 °C, 300 mg of $NaBH_4$ (7.9 mmol) was added and stirred for 2 h. The reaction mixture was diluted to 110 mL with ice−water and loaded onto a DEAE column (2.5 × 25 cm) equilibrated with 0.1 M ammonium acetate (NH_4OAc), pH 7.0. The column was washed with 500 mL of 0.1 M NH_4OAc followed by a 1 L linear gradient from 0.1 to 0.4 M NH_4OAc, pH 7.0, containing 20 mM mercaptoethanol, and finally washed with 500 mL of 0.4 M NH_4OAc, 20 mM mercaptoethanol. Fractions were collected during the NH_4OAc gradient and the final wash and analyzed by their UV−visible spectrum using 0.1 M KP_i buffer, pH 8.0. The crude product had an A_{290}/A_{245} ratio of 3.60 (76% crude yield). After lyophilization, the solid was rechromatographed on DEAE cellulose using a 1 L gradient from 0.1 to 0.4 M NH_4OAc. The pooled fractions were lyophilized, and the off-white solid was then dissolved in 5 mM mercaptoethanol and chromatographed on a cellulose column (2.5 × 50 cm) using 5 mM mercaptoethanol in water for elution. The UV−visible spectrum of the final product, after cellulose chromatography and lyophilization, had an A_{290}/A_{245} ratio >3.6. The degree of methylation of H_4folate was estimated from the integration of the ^{1}H NMR resonances for N^5-CH_3 (doublet centered at δ = 2.56 ppm, J_{CH} = 138 Hz), and the integration of the aromatic resonances of the *p*-aminobenzoyl moiety of H_4-folate (doublets centered at $\delta_{2',6'}$ = 6.70 ppm and $\delta_{3',5'}$ = 7.60 ppm), and found to be ∼80%. The ^{13}C NMR spectrum (128 scans) of a 6.0 mM solution of the ^{13}C-enriched CH_3-H_4folate in D_2O, pD 5.10, exhibited a single resonance at

44.0 ppm, indicating that methylation occurred at a single site in H_4folate. This chemical shift value agrees with the previously reported values (*23*, *24*). The proton-coupled ^{13}C NMR spectrum of the same sample exhibited a single quartet with $J_{CH} = 130$ Hz.

(6*R*)-$^{13}CH_3$-H_4folate was synthesized by the same procedure described above, except that 100 mg of (6*R*)-H_4folic acid, 0.35 mL of 20% H^{13}CHO (2.1 μmol), and 80 mg of $NaBH_4$ (2.1 μmol) were used. The final yield of the methylated substrate was 33% with an A_{290}/A_{245} ratio of 3.65. The ^{13}C NMR spectrum of a 1.25 mM solution in 20% D_2O/80%H_2O, pH 7.6, exhibited a single resonance at 42.2 ppm. The ^1H NMR spectrum of a sample in D_2O exhibited a doublet centered at 2.60 ppm with $J_{CH} = 140$ Hz. Based on the ratios of integration for this signal and that of the aromatic *p*-aminobenzoyl moiety, the product was judged to be >90% methylated.

Enzyme Preparation. MeTr expressed from a recombinant *E. coli* strain containing the MeTr gene (*6*) was purified in an anaerobic glovebox from Vacuum Atmospheres (Hawthorne, CA) at 18 °C using a protein purification liquid chromatography system from Waters. An 8 L culture of the recombinant *E. coli* strain was grown in LB medium in a shaker oven at 37 °C for 4 h until it reached an OD_{600} of 0.70. The medium contained 40 mg/L methionine, 50 mg/L ampicillin, and 35 mg/L chloramphenicol. Isopropylthiogalactoside (IPTG) was then added to a concentration of 0.8 mM. The induced cultures were grown for 3 h, and the cells were harvested by centrifugation at 10 000 rpm for 15 min at 4 °C. The *E. coli* cells (25 g) were then suspended in 100 mL of lysis buffer (50 mM Tris, pH 7.60, containing 2 mM DTT, 1 mg/mL lysozyme, 1 IU/mL DNase I, 0.1 mg/mL phenylmethylsulfonyl fluoride) at 18 °C for 1 h. After sonication for 15 min at 4 °C, the broken cells were ultracentrifuged for 90 min at 32 000 rpm at 4 °C using a Type 35 rotor from Beckman. The cell-free extract was decanted, solid $(NH_4)_2SO_4$ was added to a concentration of 0.9 M, and the solution was then heated for 45 min at 65 °C in a shaking water bath. After centrifugation at 10 000 rpm for 15 min, the supernatant was immediately loaded onto a 500 mL Phenyl Sepharose column that had been equilibrated with 0.9 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 7.60, 2 mM DTT. A linear 2.5 L gradient from 0.9 to 0.3 M $(NH_4)_2SO_4$ was developed at a flow rate of 5 mL/min. Fractions containing MeTr were identified by Western hybridization using anti-MeTr antibodies (*5*) and SDS−PAGE electrophoresis using Coomassie Brilliant Blue 250 for staining. MeTr eluted at ~0.5 M $(NH_4)_2SO_4$ and was >95% pure as judged by SDS electrophoresis. The purified enzyme (300 mg) had a specific activity of 130 nmol min^{-1} mg^{-1} in the standard assay at 55 °C with methylcobalamin and H_4folate as substrates (*18*). The concentration of protein was determined by the Rose Bengal method (*23*).

NMR Sample Preparation and NMR Experiments. All NMR spectra were acquired on a GE Omega 500 MHz NMR spectrometer at 25 °C. The ^{13}C NMR spectra were acquired at 125.75 MHz using a 45° flip observe pulse (7.25 μs pulse width) with broadband proton decoupling using the GARP1-6 modulation scheme at a power of 60 dB. The acquisition time was 0.54 s, and the relaxation delay was 1.0 s per scan. Samples for ^{13}C NMR used for T_1 measurements were prepared in 10 mm wide glass tubes from Wilmad Glass Co. (Buena, NJ). These experiments employed a T1IR.S pulse sequence (180°−τ−90°−observe). The 180° inversion pulse was determined to be 45 μs, the spectral width was set at 9520 Hz, and the pulse delay was set at 15.0 s. Two hundred scans were collected for free CH_3-H_4folate at each relaxation delay (0.1, 0.25, 0.5, 1, 2, 5, and 15 s), while 400 scans were collected for MeTr-bound CH_3-H_4folate at each relaxation delay (0.005, 0.1, 0.2, 0.5, 1, 2, and 5 s). Samples for T_1 experiments were prepared in 0.4 M MES in D_2O, pD 6.60. MeTr buffer exchange was performed by 3 cycles of concentration and dilution with MES buffer using Nanosep-10 microcentrifuge concentrators (Pall Filtron). $^{13}CH_3$-H_4folate was dissolved in MES buffer in D_2O. To measure the chemical shift and the line widths of free and MeTr-bound CH_3-H_4folate, the samples were prepared in 5 mm glass tubes (Wilmad) in 0.4 M MES buffers in D_2O or in 15% D_2O/85% H_2O. Acetone (~150 mM) was added as a chemical shift reference reagent ($\delta = 29.8$ ppm) to all samples. The pH or pD values were measured anaerobically with a portable glass electrode. In some cases, the pH was adjusted with a few microliters of 1 M HCl and 5 M NaOH. A total of 2400 scans were collected for free $^{13}CH_3$-H_4folate and 3600 scans for the MeTr-bound form with an acquisition time of 0.43 s and a pulse delay of 1.0 s. Line shape analysis was performed by fitting the $^{13}CH_3$-H_4folate signals to Lorentzian functions with the program Win-NUTS (Acorn NMR Inc., Fremont, CA). The pH and pD dependencies of the chemical shift resonances were fitted according to eq 2, where δ_{low} and δ_{high} are the limiting chemical shift resonances for protonated and unprotonated N^5-CH_3-H_4folate, and pL is either pH or pD.

$$\delta_{obs} = \frac{\delta_{low}10^{-pL} + \delta_{high}10^{-pKa}}{10^{-pL} + 10^{-pKa}} \qquad (2)$$

Proton Uptake Experiments. Proton uptake titrations were performed on a modified Cary-14 spectrophotometer from OLIS (On-Line Instrument Systems Inc., Bogart, GA) at 25 °C. The temperature of the reaction was controlled by a circulating water bath. The titrations were performed following the procedures previously outlined for cobalamin-dependent methionine synthase (*24*) and D-amino acid oxidase (*25*). All titrations were performed anaerobically using a slight overpressure of nitrogen gas in order to avoid absorption of carbon dioxide. MeTr was dialyzed 2 times with 300 volumes of 0.2 M NaCl containing 50 μM MES, pH 6.10. An aliquot of the dialyzed MeTr stock solution was added to 500 μL of a mixture of buffer and pH-indicator dye containing 50 μM MES (pK_a 6.1) and 30 μM Chlorophenol Red (p$K_a = 5.90$, $\lambda_{max} = 575$ nm, $\Delta\epsilon_{575} = 46$ mM^{-1} cm^{-1}) (*26*). The pH was adjusted with 10 mM NaOH or 10 mM HCl, and the initial absorbance at λ_{max} (575 nm) was used to calculate the initial pH for each titrated sample. Identical samples were titrated in parallel either with 10 mM NaOH or with 3.0 mM CH_3-H_4folate. The solution containing MeTr, MES, and Chlorophenol Red was titrated with NaOH to determine the proton uptake stoichiometry. The values for ΔA_{575}/mM H^+ were 4.50 and 7.5 with 36 μM MeTr monomers at pH 5.80 and pH 5.40, respectively. Stock solutions of (6*R*,*S*)-CH_3-H_4folate were prepared by dissolving the calcium salt of CH_3-H_4folate into 1 mL of the solution containing Chlorophenol Red and MES buffer. A few

microliters of 1 M HCl were added to adjust the pH so that the absorbance values at 575 nm of the MeTr and CH_3-H_4-folate solutions were identical. Then, aliquots of the CH_3-H_4folate solution were used to titrate MeTr. The amount of proton uptake associated with CH_3-H_4folate binding to MeTr was calculated from the extinction coefficients determined from the NaOH titration. Since the (6*R*,*S*)-CH_3-H_4folate concentration ranged from the same to about 4-fold higher than the MeTr concentration, the titration curves were fit to a quadratic saturation function, as shown by eq 3:

$$\frac{\Delta[H^+]}{E_t} = \frac{-b - \sqrt{b^2 - 4ac}}{2}$$

$$a = \left(\frac{[H^+] + K_{a_B}}{[H^+]}\right)^2$$

$$-b = (1 + L_t/E_t)\left(\frac{[H^+] + K_{a_B}}{[H^+]}\right) + (K_{d_U}/E_t)(K_{a_B}/K_{a_F})\left(\frac{K_{a_F} + [H^+]}{[H^+]}\right)$$

$$c = L_t/E_t \qquad (3)$$

where E_t is the total MeTr concentration, L_t is the total concentration of (6*R*,*S*)-CH_3-H_4folate, K_{d_U} is the dissociation constant for unprotonated CH_3-H_4folate, K_{a_F} and K_{a_B} are the acid dissociation constants for free and MeTr-bound CH_3-H_4folate, and $\Delta[H^+]/E_t$ is the ratio of proton uptake per MeTr monomer. Data analysis for all the experiments was performed with the program Sigmaplot for Windows (Jandel Scientific, San Rafael, CA). Our titration data cannot determine which protonation state at N^5 of CH_3-H_4folate in solution is the preferred substrate. Due to the required weak buffering conditions, proton uptake could either arise from protonation of unprotonated CH_3-H_4folate bound to MeTr or from binding of protonated CH_3-H_4folate, which causes the protonation equilibrium of free CH_3-H_4folate to shift toward the free protonated form. We therefore fitted the titration data to a mechanism where both protonated and unprotonated CH_3-H_4folate are assumed to bind to MeTr as shown by eq 4:

$$\begin{array}{ccc} \text{MeTr} + H^+ + \text{CH}_3\text{-H}_4\text{folate} & \underset{K_{d_U}}{\rightleftharpoons} & \text{MeTr–CH}_3\text{–H}_4\text{folate} + H^+ \\ K_{a_F} \updownarrow & & K_{a_B} \updownarrow \\ \text{MeTr} + \text{CH}_3\text{-H}_4\text{folate-H}^+ & \underset{K_{d_P}}{\rightleftharpoons} & \text{MeTr–CH}_3\text{-H}_4\text{folate-H}^+ \end{array} \qquad (4)$$

This treatment, which was used in the derivation of eq 3, can also be applied to the case where only one of the two protonation states of CH_3-H_4folate is the favored substrate. The dissociation constant for protonated CH_3-H_4folate from MeTr can thus be calculated from the relationship shown in eq 5, which is derived from eq 4:

$$K_{d_P} = K_{d_U}\frac{K_{a_B}}{K_{a_F}} \qquad (5)$$

Binding of CH_3-H_4folate to MeTr at pH 7.60. The determination of the dissociation constants of (6*S*)-, (6*R*)-, and (6*R*,*S*)-CH_3-H_4folate was performed in a Olis RSM-1000 (Bogart, GA) spectrophotometer−spectrofluorometer, equipped with a Scandisk operating at 60 Hz for rapid scanning. Solutions of 2.80 μM MeTr monomers were prepared anaerobically under argon in 2.0 mL of 0.1 M Tris buffer, pH 7.60. The excitation wavelength was 295 nm, and 125 scans of emission spectra were collected between 300 and 450 nm for each successive addition of substrate. The fluorescence scans were averaged, and the average at 360 nm was corrected for dilution, normalized, and plotted versus total CH_3-H_4folate added (shown in Figure 6). Equation 6 was used for fitting the fluorescence titrations with two hyperbolic dependencies.

$$F = \frac{\Delta F_1[L_t]}{[L_t] + K_{d_1}} + \frac{\Delta F_2[L_t]}{[L_t] + K_{d_2}} + F_i \qquad (6)$$

Binding of (6R,S)-CH_3-H_4folate to MeTr Measured by Equilibrium Dialysis. The measurement of the binding of CH_3-H_4folate to MeTr was carried out in the anaerobic glovebox by the method used by Jarrett for methionine synthase (*24*). In a typical set of experiments at a given pH, a solution of 37−40 μM MeTr monomers was prepared in the inverted cap of a 0.5 mL microfuge tube. This was then covered with a small piece of dialysis tubing (12−14 kDa cutoff, Spectra/Por, Spectrum Medical, Laguna Hills, CA), which was previously soaked in the same buffer used later for dialysis. The bottom part of the microfuge tube, from which a small hole was previously cut, was put on the microfuge cap with the dialysis tubing and MeTr solution. Then buffer and radiolabeled (6*R*,*S*)-CH_3-H_4folate were added on top of the dialysis tubing (200 μL). The stock substrate solution was a mixture of ^{14}C- and ^{12}C-labeled (6*R*,*S*)-CH_3H_4folate with a specific radioactivity of ~5000 dpm/nmol. The assumption was made that $^{14}CH_3$-H_4folate, which was chemically prepared, consisted of equal amounts of the two diastereomers. The samples were then covered with Parafilm paper and incubated for 16−24 h, after which the Parafilm paper was removed and the microfuge tubes were inverted into the bottom part of 1.5 mL microfuge tubes. The solution containing free CH_3-H_4folate was removed by centrifugation for 30 s at 7000 rpm, and 50 μL of this solution was transferred using a Hamilton syringe into a scintillation vial. The solution containing free plus MeTr-bound CH_3-H_4folate (still inside the cap of the 0.5 mL microfuge tube) was removed by puncturing the dialysis membrane with the syringe and transferring 50 μL of this solution into a separate scintillation vial. The radioactivity of the solutions was measured in a Packard Tri-Carb Scintillation Analyzer. The concentrations of free and free plus bound CH_3-H_4folate were calculated according to eqs 7 and 8:

$$[\text{CH}_3\text{-H}_4\text{folate}]_{\text{free}} = \frac{\text{dpm}_S}{\text{SA}(0.050\ \text{mL})} \qquad (7)$$

$$[\text{CH}_3\text{-H}_4\text{folate}]_{\text{bound}} = \frac{\text{dpm}_E - \text{dpm}_S}{\text{SA}(0.050\ \text{mL})} \qquad (8)$$

where SA is the specific activity (5000−5200 dpm/nmol)

of (6*R*,*S*)-CH_3-H_4folate, dpm_S are the counts for the side without MeTr, and dpm_E are the counts for the side with MeTr. The dissociation constant was obtained by plotting the concentration of MeTr-bound CH_3-H_4folate versus free CH_3-H_4folate and fitting to simple dissociation curves at each pH (Figure 6).

RESULTS

^{13}C NMR of Free CH_3-H_4folate. The position and line width of the ^{13}C resonance for the N^5-methyl group of $^{13}CH_3$-H_4folate are pH dependent (*27*). We recorded the spectrum of ^{13}C-enriched CH_3-H_4folate at pD values between 3.4 and 6.8 to assess the feasibility of using NMR to diagnose the protonation state of enzyme-bound CH_3-H_4folate. The pD dependence (not shown) was fitted to a single titration curve (eq 2), which gave the following values: δ_{low} of 44.53 $\pm$ 0.06 ppm, δ_{high} of 42.41 $\pm$ 0.07 ppm, and pK_a(D_2O) of 5.32 $\pm$ 0.06. Since the pK_a in H_2O for the N^5-amine group of (6*R*,*S*)-CH_3-H_4folate is 4.82−4.88 (*19*, *12*), this result indicates that the protonated amino group is 3 times less acidic in D_2O than in H_2O. This is consistent with proton transfer from protonated CH_3-H_4folate to water forming a hydronium ion. The calculated fractionation factor for protonated CH_3-H_4folate is then ~1.0 (*28*). Protonation of CH_3-H_4folate shifts the $^{13}CH_3$ resonance downfield by about 260 Hz, presumably due to an inductive effect and a decrease in electron density at N^5, which leads to deshielding of the N^5-methyl carbon.

The line width of the resonance broadens significantly upon lowering the pD, from 4 Hz at pD 6.8 to 47.5 Hz at pD 3.5. Line shape analysis of this signal shows that the peaks are Lorentzian, indicating that the line width is not affected by field inhomogeneities. Thus, the line width can be used as a measure of the transversal relaxation time T_2 ($\Delta\nu_{1/2} = 1/\pi T_2$) for the methyl group, which varies from 80 ms at pD 6.8 to 6.7 ms at pD 3.5. The value of the longitudinal relaxation time (T_1) at a pD of 6.6 was determined to be 0.71 $\pm$ 0.01 s.

^{13}C NMR of MeTr-Bound CH_3-H_4folate. Previous equilibrium titrations of MeTr with (6*S*)-CH_3-H_4folate showed that upon binding, this substrate quenches the fluorescence of MeTr at pH 7.60 (*18*). Figure 1 shows the results of the titration of (6*R*,*S*)-$^{13}CH_3$-H_4folate with MeTr at pH 7.60, using acetone as a chemical shift reference standard in 20% D_2O in water. When the ratio of ^{13}C-labeled substrate to enzyme monomers varied from infinite (no MeTr present) to 9:1, only one signal was detected (after 2400 scans) at the chemical shift of free $^{13}CH_3$-H_4folate (δ = 42.24 ppm) (spectrum A). Although this signal does not broaden (line width 4−5 Hz), the signal intensity decreases significantly as the relative amount of MeTr is increased, suggesting that binding to MeTr is occurring. This is independently confirmed by measuring, at the same pH, the dissociation constant of (6*R*,*S*)-$^{13}CH_3$-H_4folate by equilibrium dialysis (9 μM) and fluorescence quenching (13 μM) (vide infra). When more MeTr is added to $^{3}CH_3$-H_4folate, a second signal appears with chemical shifts ranging from δ = 42.80 ppm (spectrum B) to δ = 42.61 ppm (spectrum F) and line widths ranging from 25 to 40 Hz. The signal centered at δ = 42.2 ppm, which we assume corresponds to the free cofactor, decreases in intensity, but remains at the same chemical shift and has the same line width (4−5 Hz) as the signal for the free $^{13}CH_3$-H_4folate (spectrum A). When the MeTr concentration is increased to approach the total concentration of (6*R*,*S*)-$^{13}CH_3$-H_4folate, the signal for free $^{13}CH_3$-H_4folate is replaced by a broader signal (line width 22 Hz) centered at δ = 42.0 ppm (spectrum G).

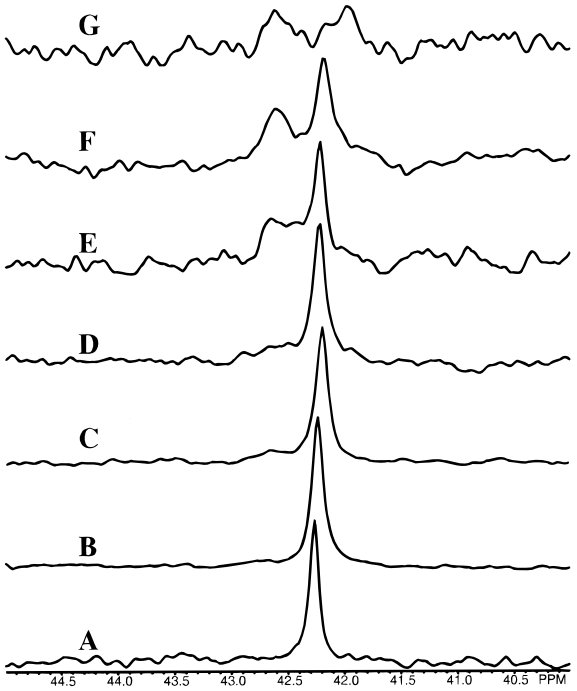

FIGURE 1: ^{13}C NMR spectrum of the titration of (6*R*,*S*)-$^{13}CH_3$-H_4folate with MeTr. Samples were prepared in 20% D_2O/80% H_2O with 0.05 M, Tris buffer, pH 7.60. Spectra were collected with a data acquisition time of 0.43 s and a pulse delay of 1.0 s per scan, and the line shape analysis was performed with the program WinNUTS. The ratios of (6*R*,*S*)-$^{13}CH_3$-H_4folate to MeTr and the concentrations of (6*R*,*S*)-$^{13}CH_3$-H_4folate added were as follows: spectrum A, infinite (no MeTr) and 3.22 mM; B, 9.1 and 2.23 mM; C, 6.0 and 1.93 mM; D, 4.0 and 1.34 mM; E, 2.6 and 1.09 mM; F, 1.80 and 1.10 mM; G, 1.25 and 0.83 mM. Other conditions are described under Materials and Methods.

Folate-dependent enzymes typically bind only one of the diastereomers of H_4folate [usually the (6*S*) form]. Since these experiments were performed with the *(6R,S)* mixture, we expected to observe the ^{13}C resonance for free (6*R*)-$^{13}CH_3$-H_4folate as a constant background at 42.2 ppm. Instead, as the relative concentration of MeTr to (6*R*,*S*)-$^{13}CH_3$-H_4folate approaches a 1:1 ratio, two broad signals are observed (spectrum G), and the peak assigned to the free cofactor decreases and finally disappears. Thus, we hypothesized that MeTr can bind both stereoisomers. To test if (6*R*)-$^{13}CH_3$-H_4folate binds to MeTr, we performed ^{13}C NMR spectroscopy of samples with ratios of (6*R*)-$^{13}CH_3$-H_4folate to MeTr monomers of 3:1 (Figure 2A), 2:1 (Figure 2B), and 1:1 (Figure 2C), respectively. The signal is much broader (line widths between 37 and 41 Hz) than that of free (6*R*)-$^{13}CH_3$-H_4folate (4 Hz, not shown). The signal also moves away from the chemical shift of free $^{13}CH_3$-H_4folate (42.20 ppm) as more MeTr is added. The spectrum for the sample with a one-to-one ratio (spectrum C in Figure 2) could be fitted assuming that there are two peaks centered at 41.75 and 42.23 ppm with relative areas of 4 to 1, respectively. The peak at 42.23 ppm is at the same chemical shift as free (6*R*,*S*)-

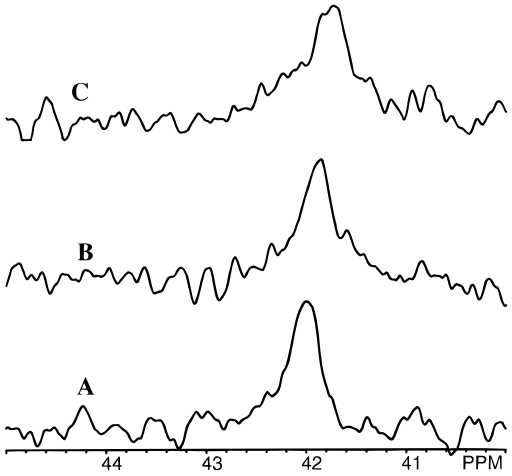

FIGURE 2: ^{13}C NMR spectrum of the titration of (6*R*)-$^{13}CH_3$-H_4-folate with MeTr. Samples were prepared in 20% D_2O/80% H_2O in 0.05 M Tris buffer, pH 7.60, at 23 °C. Spectra were collected with a data acquisition time of 0.43 s and a pulse delay of 1.0 s per scan, and the line shape analysis was performed with the program WinNUTS. The ratios of (6*R*)-$^{13}CH_3$-H_4folate to MeTr and the (6*R*)-$^{13}CH_3$-H_4folate concentrations used were as follows: spectrum A, 3.0 and 1.77 mM; B, 2.0 and 1.20 mM; C, 1.0 and 0.62 mM. Other conditions are described under Materials and Methods.

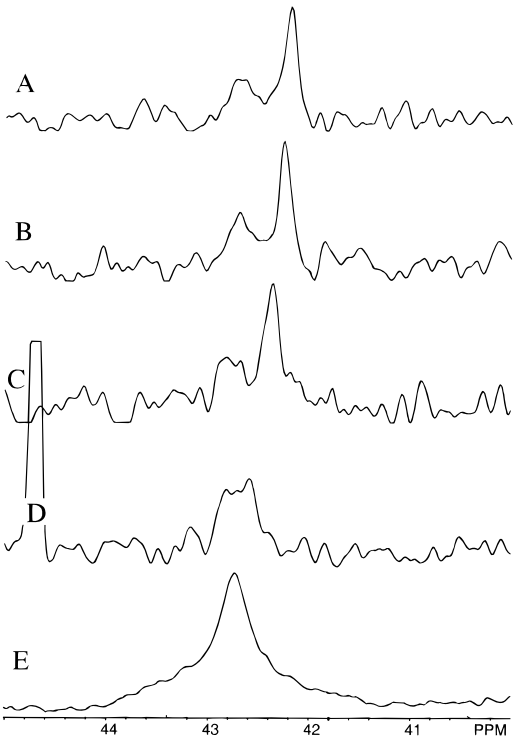

FIGURE 3: ^{13}C NMR spectrum of MeTr with (6*R,S*)-$^{13}CH_3$-H_4folate. Mixtures of 1 mM ^{13}C-enriched (6*R,S*)-CH_3-H_4folate and 1 mM MeTr were prepared in 0.2 M MES buffers in D_2O. A total of 3600 scans were collected per spectrum with a data acquisition time of 0.43 s and a pulse delay of 1.0 s per scan at 23 °C. Other conditions are described under Materials and Methods. The spectra correspond to the following pD values: A, 7.60; B, 7.00; C, 6.50; D, 6.00; E, 5.30.

$^{13}CH_3$-H_4folate and free (6*R*)-$^{13}CH_3$-H_4folate ($\delta = 42.2$ ppm). The observed peak ratio indicates that 80% of the (6*R*) diastereomer is bound: thus, its dissociation constant from MeTr is about 20 μM. At higher concentrations of the (6*R*) diastereomer, only one signal is observed, in stark contrast with the spectra in Figure 1. Since the spectra for Figures 1 and 2 were acquired under nearly identical conditions, the observed signals in Figure 2A,B must be comprised of both the free and MeTr-bound (6*R*)-$^{13}CH_3$-H_4folate. Moreover, the signals for the sample with 830 μM (6*R,S*)-$^{13}CH_3$-H_4folate and 670 μM MeTr monomers, shown in Figure 1G, must correspond to MeTr-bound (6*S*)-$^{13}CH_3$-H_4folate ($\delta = 42.58$ ppm) and MeTr-bound (6*R*)-$^{13}CH_3$-H_4folate ($\delta = 42.0$ ppm). That the (6*R*) isomer binds to MeTr is confirmed by equilibrium dialysis and fluorescence quenching experiments (vide infra).

To determine the protonation state of the MeTr-bound substrate, we acquired ^{13}C NMR spectra (Figure 3) of samples containing 1 mM (6*R,S*)-$^{13}CH_3$-H_4folate and 1 mM MeTr monomers, since such samples must exhibit the two signals for bound $^{13}CH_3$-H_4folate, and subjected the data to line shape analysis. As the pH is lowered, the two signals shown in Figure 3 move closer to each other, and, below a pD of 6.0, they coalesce into a broad signal. Such samples containing 15% D_2O in water exhibit only one broad signal at pH values below 5.90 (not shown). The pD and pH dependencies of the chemical shift for the resonances are shown in Figure 4 (closed circles, D_2O; open circles, H_2O), and the parameters obtained from the line shape analysis were fit to eq 2. The results in Table 1 clearly show that binding of $^{13}CH_3$-H_4folate to MeTr increases the pK_a for the N^5 tertiary amine by 1 p*K* unit in D_2O. Since the pK_a for free $^{13}CH_3$-H_4folate is raised by 0.5 p*K* unit in D_2O as compared with 15% D_2O in water, then the pK_a for $^{13}CH_3$-H_4folate in 15% D_2O is a full p*K* unit higher than free in solution. Both

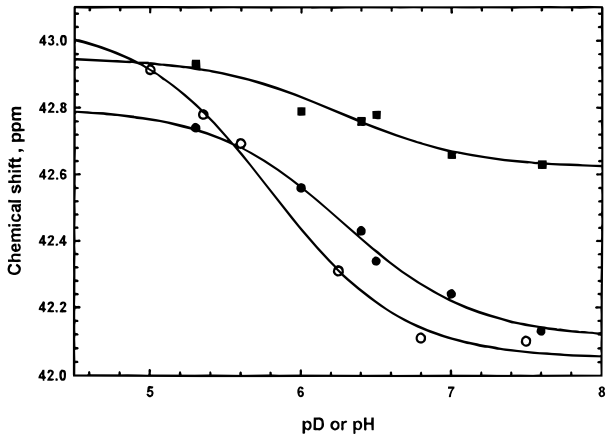

FIGURE 4: Dependence of the observed chemical shift resonance on the pH and pD. The ^{13}C NMR signals for MeTr-bound (6*R,S*)-$^{13}CH_3$-H_4folate were fitted using the program Win-NUTS and plotted here. Closed circles are for the fitted chemical shifts for high-field and low-field ^{13}C signals in D_2O. Open circles are for the chemical shifts for the high-field signal in 15% D_2O in H_2O, closed circles for the high-field signal in D_2O, and closed squares for the low-field signal in D_2O.

the (6*R*) and (6*S*) diastereomers exhibit this elevated pK_a upon binding to MeTr (Table 1).

The T_1 values for the two signals for bound $^{13}CH_3$-H_4-folate at a pD of 6.6 were 0.93 ± 0.05 s for the low-field signal and 0.75 ± 0.04 s for the high-field signal. The line widths of the MeTr-bound signals are pH-independent, unlike those for free $^{13}CH_3$-H_4folate. The average line widths for

Table 1: pH and pD Dependencies of the NMR Resonance of Free and MeTr-Bound $^{13}CH_3$-H_4folate[a]

	chemical shift (ppm)		
	protonated CH_3-H_4folate	unprotonated CH_3-H_4folate	pK_a
Free CH_3-H_4folate			
D_2O signal	44.53 ± 0.06	42.41 ± 0.07	5.32 ± 0.06
MeTr-Bound CH_3-H_4folate			
D_2O low-field signal	42.95 ± 0.05	42.62 ± 0.04	6.26 ± 0.25
D_2O high-field signal	42.80 ± 0.03	42.11 ± 0.03	6.28 ± 0.08
H_2O signal[b]	43.05 ± 0.05	42.05 ± 0.03	5.80 ± 0.09[c]

[a] The concentrations used were 1 mM CH_3-H_4folate in D_2O and 1 mM CH_3-H_4folate both in 15% D_2O in H_2O and in D_2O. Below a pD or a pH of 5.9, only one broad resonance is observed (Figure 3), which can, nevertheless, be fitted to two signals for the samples in D_2O. [b] In H_2O, the two signals were assumed to be at the same chemical shift below pH 6.0, but only the upfield signal was plotted against pH in Figure 4. [c] The calculated solvent isotope effect for the upfield resonance is 3.01.

the MeTr-bound $^{13}CH_3$-H_4folate signals, over the pD range covered in Figure 3, are 20 ± 7 Hz for the high-field signal and 22 ± 7 Hz for the low-field resonance. This corresponds to 5- and 6-fold broadening relative to free CH_3-H_4folate, respectively.

Proton Uptake upon CH_3-H_4folate Binding to MeTr. The ^{13}C NMR experiments demonstrate that binding of CH_3-H_4folate to MeTr increases the basicity of CH_3-H_4folate by 10-fold ($K_{a_B}/K_{a_F} = 10$, Table 1). This suggested that binding of CH_3-H_4folate to MeTr could result in proton uptake. When we added CH_3-H_4folate to MeTr in the presence of the pH indicator Chlorophenol Red, we observed an increase in absorbance at 575 nm, which indicates proton uptake as the binary complex MeTr−CH_3-H_4folate is formed. The value of pK_{a_B} (acid dissociation constant for the binary complex) was calculated from the initial slope of proton uptake (protons per CH_3-H_4folate bound) and from the amplitude of the full titration (protons per MeTr sites) (Table 2). The obtained pK_{a_B} values are between 5.50 and 6.00, in agreement with the ^{13}C NMR results. The titrations were fitted to the quadratic binding eq 3, and the data for three of the titrations are shown in Figure 5. These experiments also demonstrate that both monomers in the MeTr homodimer are able to bind (6*R*,*S*)-CH_3-H_4folate and that the proton that is taken up causes development of positive charge at the N^5 group of the pterin ring. Presumably protonation occurs directly at the N^5 position; however, scenarios in which protonation at another site, e.g., C-8, leads to positive charge development at N^5 are also possible. Titrations with (6*R*,*S*)-H_4folate (not shown) also yielded pK_{a_B} values for H_4folate between 5.50 and 6.00 and a stoichiometric binding of 1 H_4folate per MeTr monomer. Thus, MeTr increases the basicity of both H_4folate substrates upon binding.

Fluorescence Quenching Titrations of MeTr with CH_3-H_4folate. Given the unusual finding that both diastereomers of CH_3-H_4folate bind to MeTr, we determined the relative affinity of MeTr for (6*S*)- and (6*R*)-CH_3-H_4folate by titrating MeTr with the individual diastereomers and with the mixture of (6*R*,*S*)-$^{13}CH_3$-H_4folate. The titration was followed by measuring the quenching of intrinsic tryptophan fluorescence (Figure 6), and the data were fitted to a two binding site equation. The titration with (6*S*)-CH_3-H_4folate yielded dissociation constants (2.1 and 65 μM) similar to those previously measured in our laboratory (*18*). The titrations with (6*R*,*S*)-CH_3-H_4folate yielded similar dissociation constants as with (6*S*)-CH_3-H_4folate alone. When the fluorescence quenching data for the (6*R*) diastereomer were fitted to the same equation, the dissociation constants were nearly identical (26 μM), but the error was higher than 100%, indicating identical binding sites. When the data were fit to a one-site binding curve, the dissociation constant was 26 μM and the error significantly improved. These results clearly show that MeTr possesses equivalent binding sites for the (6*R*) diastereomer and nonequivalent binding sites for the (6*S*) diastereomer, and they suggest that two isomers bind independently.

Equilibrium Dialysis Experiments. Fluorescence titrations cannot yield the pH dependence of the dissociation constants for CH_3-H_4folate, because the protonated form of CH_3-H_4folate is much more fluorescent than the unprotonated form and interferes with the fluorescence of MeTr (*18*). Thus, we determined the dissociation of (6*R*,*S*)-CH_3-H_4folate from the binary complex with MeTr by equilibrium dialysis at several pH values, and fitted the results to single binding curves at each pH (Table 3). As Figure 7 clearly shows, the binding data at four different pH values could be fitted to a single binding curve with a K_d of 10 ± 1 μM. If the fluorescence titration data with (6*R*,*S*)-CH_3-H_4folate are also fitted to a single binding curve, an apparent K_d of 9.6 ± 0.8 μM is obtained (not shown), thus showing close agreement between these two different experiments. Proton uptake experiments at pH 7.60 are not possible to perform because the bound substrate remains unprotonated, but the K_d values (Table 2) are in agreement with those from equilibrium dialysis (Table 3).

DISCUSSION

The studies described here provide strong evidence that binding of CH_3-H_4folate to MeTr result in transfer of a proton

Table 2: Proton Uptake Titrations for MeTr with CH_3-H_4folate

pH[a]	slope, H^+ per CH_3-H_4folate	calculated[b] pK_{a_B}	uptake, H^+ per MeTr	calculated[b] pK_{a_B}	CH_3-H_4folate[c] bound per MeTr	calculated Kd_U, μM
6.80	0.150 ± 0.013	5.85	0.256 ± 0.010	6.14	1.2 ± 0.2	19 ± 7
6.50	0.150 ± 0.015	5.75	0.240 ± 0.020	6.00	1.06 ± 0.1	9 ± 5
5.80	0.42 ± 0.08	5.65	0.360 ± 0.002	5.55	0.80 ± 0.06	5 ± 2
5.40	0.68 ± 0.13	5.74	0.665 ± 0.002	5.72	0.93 ± 0.05	11 ± 2
5.20	0.73 ± 0.15	5.63	0.84 ± 0.14	5.92	1.20 ± 0.05	58 ± 10

[a] Refers to the initial pH of the titration; during a given titration, the pH did not increase by more than 0.25 pH unit. [b] Values of pK_{a_B} shown in the third column are calculated from the proton uptake per CH_3-H_4folate from the first data points in the titration, while those shown in the fifth column are calculated from the limiting value of proton uptake at saturating CH_3-H_4folate per MeTr monomer. [c] The last two columns were calculated from eq 3 in the text.

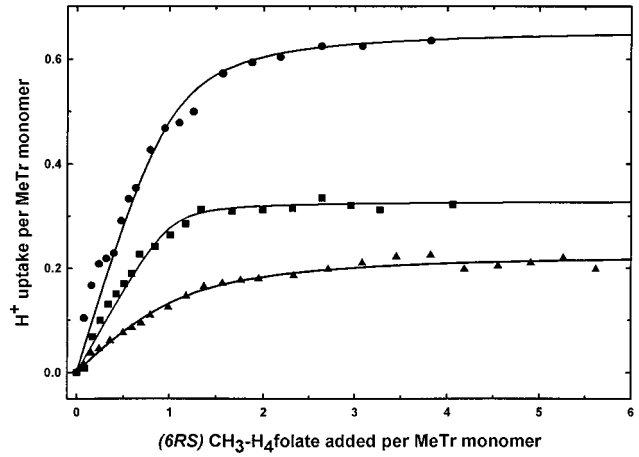

FIGURE 5: Proton uptake by MeTr upon titration with (6*R*,*S*)-CH_3-H_4folate. Absorbance changes were monitored at 575 nm for the acid−base dye Chlorophenol Red, and converted to proton uptake per MeTr monomer as outlined under Materials and Methods. Data are for titrations at an initial pH of 5.40 (closed circles), 5.80 (closed squares), and 6.60 (close triangles). Curves are fits to eq 3 using the parameters shown in Table 2.

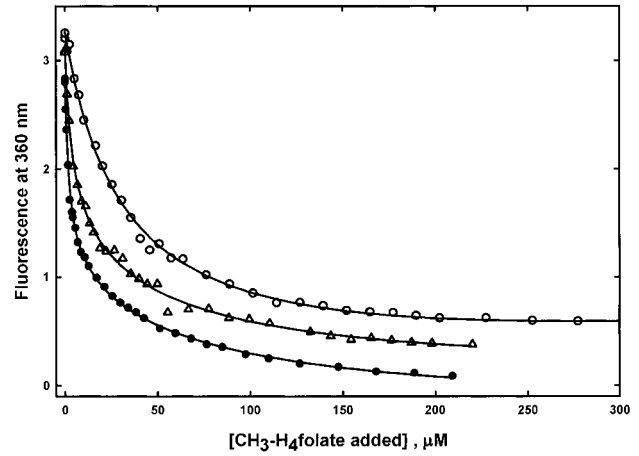

FIGURE 6: Binding of CH_3-H_4folate to MeTr at pH 7.60. The substrate was anaerobically added to a solution of 2.80 μM MeTr monomers in 100 mM Tris, pH 7.60. The tryptophan fluorescence was obtained by exciting the sample at 295 nm and measuring the emission intensity at 360 nm. Data for (6*S*)-CH_3-H_4folate are shown as closed circles, for (6*R*)-CH_3-H_4folate as open circles, and for the mixture of both diastereomers (equal concentrations of each) as open triangles. Curves are fits to eq 8 in the text with following the parameters for (6*S*)-CH_3-H_4folate: $\Delta F_1 = 1.78 \pm 0.06$, $K_{d_1} = 2.1 \pm 0.18$ μM, $\Delta F_2 = 1.31 \pm 0.05$, $K_{d_2} = 65 \pm 11$ μM, $F_i = -0.26 \pm 0.05$; for (6*R*)-CH_3-H_4folate: $\Delta F_1 = 3.01 \pm 0.03$, $K_{d_1} = 26.2 \pm 1.2$ μM, $F_i = -0.26 \pm 0.05$ (a single-site titration hyperbola); for (6*R*,*S*)-CH_3-H_4folate: $\Delta F_1 = 1.65 \pm 0.24$, $K_{d_1} = 3.6 \pm 0.9$ μM, $\Delta F_2 = 1.37 \pm 0.19$, $K_{d_2} = 50 \pm 18$ μM, $F_i = -0.17 + 0.05$. Data for the (6*R*) diastereomer and (6*R*,*S*) mixture are shifted upward along the *y*-axis by 0.50 and 0.25 unit, for the purpose of clarity.

from solvent to the pterin ring. This step has been hypothesized to enhance catalysis because it would lead to a positive charge on N^5, thus enhancing the electrophilicity of the bound methyl group. The first line of evidence supporting this hypothesis is provided by ^{13}C NMR studies of $^{13}CH_3$-H_4-folate bound to MeTr. We observe two peaks (Figure 1G) that we assign to two forms of MeTr-bound $^{13}CH_3$-H_4folate based on the following reasoning. First, these signals are 5-fold broader than the signal of free $^{13}CH_3$-H_4folate, and the signal for free $^{13}CH_3$-H_4folate is not broadened by

Table 3: Equilibrium Dialysis Experiments[a]

pH	maximal ligand binding per MeTr monomer	dissociation constant, μM
4.85	0.84 ± 0.06	2.1 ± 0.7
5.20	1.15 ± 0.10	2.1 ± 0.7
5.90	$1.10 + 0.10$	3.0 ± 0.80
6.20	1.07 ± 0.10	4.7 ± 1.9
6.60	1.30 ± 0.10	6.2 ± 1.7
7.60	0.86 ± 0.11	9.0 ± 4.8
8.50	1.22 ± 0.05	5.2 ± 1.2

[a] Equilibrium dialysis determinations were carried out using (6*R*,*S*)-CH_3H_4folate with $^{12}CH_3$ and $^{14}CH_3$ (as tracer) bound to N^5. See Materials and Methods for details.

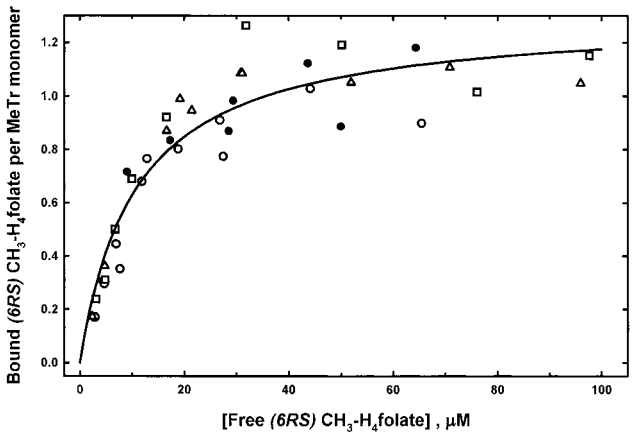

FIGURE 7: Determination of the binding constant of (6*R*,*S*)-CH_3-H_4folate to MeTr by equilibrium dialysis. The concentrations of free and MeTr-bound CH_3-H_4folate were calculated as described under Materials and Methods and normalized with respect to the MeTr monomer concentration for each determination. Data correspond to the following pH values: closed circles, 5.20; open circles, 5.90; triangles, pH 6.60; squares, 8.50. The curve is the fit to a simple binding equation with maximal binding of 1.3 CH_3-H_4folate per MeTr monomer and a $K_d = 10 \pm 1$ μM.

exchange with bound $^{13}CH_3$-H_4folate. Second, the dissociation constants for (6*S*), (6*R*), and a mixture of both diastereomers (6*R*,*S*) are much lower than the MeTr concentration present in this sample (670 μM) (as well as all other samples for which spectra are included in Figures 1 through 3). Thus, most of the substrate is bound (see spectra in Figure 3). Third, the chemical shifts and line widths for the signals that we assign as free $^{13}CH_3$-H_4folate in spectra 1B through 1E are identical to those of $^{13}CH_3$-H_4folate alone (spectrum A), but the upfield signal in spectrum G is centered at 42.0 ppm. Fourth, the pD dependencies for the two signals (Figure 3 and Table 1) yield pK_a values that are 1 pK unit higher than the pK_a for free $^{13}CH_3$-H_4folate.

The observation of two signals for MeTr-bound CH_3-H_4-folate in both protonated and unprotonated states indicates that there are two distinct bound environments. Since all of the (6*R*,*S*)-$^{13}CH_3$-H_4folate present was bound to MeTr, we suspected that MeTr was resolving the signals of the two diastereomers upon interaction with the active site. Under the conditions of the NMR titration shown in Figure 1, binding of the natural isomer (6*S*)-$^{13}CH_3$-H_4folate is favored over binding of the unnatural (6*R*) diastereomer, as indicated by the values of K_{d_1} (2.1 μM) and K_d (26 μM) (Figure 6). Therefore, the downfield signal must correspond to the MeTr-bound (6*S*)-$^{13}CH_3$-H_4folate. The chemical shift of this species

moves upfield as more MeTr is added, moving closer to the chemical shift for the resonance for free $^{13}CH_3$-H_4folate. This is the opposite direction of changes as predicted by a simple exchange mechanism between free and protein-bound ligand. However, the signal for bound (6*R*)-$^{13}CH_3$-H_4folate (Figure 2) moves downfield in chemical shift upon titration with more MeTr, and away from the resonance for free $^{13}CH_3$-H_4folate. This observation implies that free and MeTr-bound (6*R*)-$^{13}CH_3$-H_4folate are in rapid exchange. The lower degree of affinity of the enzyme for the unnatural (6*R*) relative to the (6*S*) substrate allows this exchange to be observed (low-field shoulder in Figure 2C). However, the same exchange mechanism cannot be observed with the mixture of diastereomers (Figure 1B through 1F). When both diastereomers are present, the exchange mechanism involves MeTr-bound (6*S*)-$^{13}CH_3$-H_4folate ($\delta = 42.8-42.6$ ppm in Figure 1) with MeTr-bound (6*R*)-$^{13}CH_3$-H_4folate or exchange with the (6*S*) diastereomer bound to the second MeTr site, which has a higher dissociation constant (between 50 and 65 μM, Figure 6).

The $^{13}CH_3$ resonances for protonated $^{13}CH_3$-H_4folate are shifted downfield by ~200 Hz upon interaction with MeTr. This is probably due to delocalization of the positive charge on the N^5 of the pteridine ring through a hydrogen (or deuterium) bond with the protein. In contrast, the signals for unprotonated MeTr-bound $^{13}CH_3$-H_4folate are shifted downfield by just 26 Hz (low-field signal) and upfield by just 31 Hz (high-field signal), which suggests a weaker interaction between CH_3-H_4folate and the protein active site when unprotonated than when protonated.

MeTr from *Clostridium thermoaceticum* catalyzes the methyl transfer from CH_3-H_4folate to cob(I)alamin (*32*) or to the cob(I)amide form of the CFeSP (*15*, *16*) by an S_N2 mechanism (*12*). While both of these cobalt−base species are strong nucleophiles, MeTr must still activate the tertiary amine at N^5 of CH_3-H_4folate in order for nucleophilic displacement to occur. One likely mechanism for activation is protonation at N^5 of CH_3-H_4folate to a quaternary ammonium ion, which is characterized by an increase in the basicity of CH_3-H_4folate upon interaction with MeTr (*29*). Indeed, 5,5,6,7-tetramethyl-5,6,7,8-tetrahydropteridinium tetrafluoroborate, a model for protonated CH_3-H_4folate, is able to react with cob(I)alamin to form methylcob(III)alamin and the corresponding trimethylated product (*16*, *17*). Kinetic studies with MeTr, primarily of the pH dependencies of the rate constants, were unable to establish to what extent the pK_a for the N^5 of CH_3-H_4folate was increased by binding to MeTr. The experiments using ^{13}C-enriched CH_3-H_4folate presented here demonstrate that there is an increase of 1 pK unit in both H_2O and D_2O. Upon titration of MeTr with CH_3-H_4folate or H_4folate, proton uptake was observed (Figure 5); this is consistent with an increase in the basicity at the N^5-amine in both substrates.

The results described above indicate that elevation of the pK_a might result from formation of a H-bonding interaction with the protein. We hope that the crystal structure of MeTr, which is near completion, will reveal interactions with the substrate that are responsible for this key step in catalysis.

The fact that MeTr increases the pK_a of the N^5 of CH_3-H_4folate by 1 pK unit implies that, at equilibrium, MeTr must bind protonated CH_3-H_4folate 10-fold tighter than unprotonated CH_3-H_4folate. This expectation was tested by equilibrium dialysis experiments, using a mixture of both (6*S*)- and (6*R*)-CH_3-H_4folates. Since both diastereomers undergo an increase in their corresponding N^5 pK_a values (as determined by the NMR experiments) and, consequently, lead to proton uptake upon binding to MeTr, the pH dependencies of their dissociation constants must be very similar. We find that the K_d for (6*R*,*S*)-CH_3-H_4folate is pH independent (Table 3 and Figure 7). We have demonstrated that the dissociation constant for the methyl acceptor substrate, CFeSP, is also pH independent (*30*). The results shown in Table 2 show that the ratios of proton uptake per MeTr monomer and per CH_3-H_4folate at a given initial pH are very similar. Thus, the extent of proton uptake upon formation of the binary complex is determined solely by the ionization state at N^5 of CH_3-H_4folate, and not by the ionization states at other remote sites either in CH_3-H_4folate or in MeTr. The pH-dependent conformational change of MeTr cannot, therefore, play a role in protonation of CH_3-H_4folate at the active site. We conclude that the unprotonated state of CH_3-H_4folate must be the substrate of MeTr, since it is the predominant form within the pH range at which MeTr is active. Furthermore, there is close agreement between the K_{d_U} values for unprotonated (6*R*,*S*)-CH_3-H_4folate (Table 2) and the K_d values from equilibrium dialysis experiments at variable pH (Table 3). We also conclude that the conformational change of MeTr does not modulate the binding of the substrates in the forward direction, and presumably the same must follow for the substrates in the reverse direction. This is in sharp contrast with the mechanism of binding of the extrinsic hydrophobic probe bis-ANS (*18*), which is strongly pH dependent and modulated by the protein conformational change.

The stereochemical requirements of the S_N2 displacement mechanism require that the proton at N^5 of protein-bound CH_3-H_4folate be on the opposite side of the pteridine plane from where the nucleophilic cob(I)amide approaches the methyl group. The enzyme can achieve this by binding the unprotonated form, followed by protonation at the correct orientation. Another mechanism, which was suggested to us by Rowena Matthews, is to protonate the substrate at C^8. Delocalization of electron density would still generate positive charge at N^5, which would be entirely consistent with our spectroscopic and kinetic results. Stabilization of the protonated form of the substrate could be achieved by interactions with one or more polar groups in the enzyme. Candidates would include an ionized carboxylic acid moiety, the pK_a of which would be also increased upon substrate binding.

There are several implications of the present work on the mechanism of methyl transfer. First, although MeTr binds protonated CH_3-H_4folate 10-fold stronger than unprotonated CH_3-H_4folate, the unprotonated state is the preferred substrate. Second, proton uptake occurs in the ternary complex

[2] Since the protonated state of CH_3-H_4folate is the most likely substrate, it must undergo protonation during the cycle of catalysis. This protonation must occur prior to methyl transfer, since it is observed in the MeTr−CH_3-H_4folate binary complex. Therefore, our observations of proton uptake, with a pK_a value identical to that determined by ^{13}C NMR, indicate that the event that triggers protonation is substrate binding and that protonation occurs after formation of the binary complex. Similarly, if the proton were to be provided by MeTr, no proton uptake is expected when MeTr is titrated with CH_3-H_4folate.

during catalysis. Third, binding of unprotonated CH_3-H_4folate and the proton must follow a strictly ordered pathway.[2] Fourth, MeTr inactivates the reverse reaction by protonation of MeTr-bound H_4folate due to the increase in the basicity at N^5. Fifth, the proton taken up by the MeTr-bound CH_3-H_4folate must originate in solvent, not in MeTr. These results differ from the previous studies with methionine synthase (*24*), which concluded that binding of CH_3-H_4folate occurred without the uptake of a proton, but agree with the previous observation that its K_d is pH independent (*31*).

REFERENCES

1. Ljungdahl, L. G. (1986) *Annu. Rev. Microbiol. 40*, 415−450.
2. Ragsdale, S. W. (1991) *CRC Crit. Rev. Biochem. Mol. Biol. 26*, 261−300.
3. Ragsdale, S. W., and Kumar, M. (1996) *Chem. Rev. 96*, 2515−2539.
4. Drake, H. L., Hu, S.-I., and Wood, H. G. (1981) *J. Biol. Chem. 256*, 11137−11144.
5. Roberts, D. L., James-Hagstrom, J. E., Smith, D. K., Gorst, C. M., Runquist, J. A., Baur, J. R., Haase, F. C., and Ragsdale, S. W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 32−36.
6. Roberts, D. L., Zhao, S., Doukov, T., and Ragsdale, S. W. (1994) *J. Bacteriol. 176*, 6127−6130.
7. Doukov, T., Zhao, S., Roberts, D. L., Kim, J.-J., Ragsdale, S. W., and Stezowski, J. (1995) *Acta Crystallogr. D51; Part 6*, 1092−1093.
8. Hu, S.-I., Pezacka, E., and Wood, H. G. (1984) *J. Biol. Chem. 259*, 8892−8897.
9. Ragsdale, S. W., Lindahl, P. A., and Münck, E. (1987) *J. Biol. Chem. 262*, 14289−14297.
10. Lu, W.-P., Schiau, I., Cunningham, J. R., and Ragsdale, S. W. (1993) *J. Biol. Chem. 268*, 5605−5614.
11. Menon, S., and Ragsdale, S. W. (1998) *Biochemistry 37*, 5689−5698.
12. Zhao, S., Roberts, D. L., and Ragsdale, S. W. (1995) *Biochemistry 34*, 15075−15083.
13. Harder, S. A., Lu, W.-P., Feinberg, B. F., and Ragsdale, S. W. (1989) *Biochemistry 28*, 9080−9087.
14. Wirt, M. D., Kumar, M., Ragsdale, S. W., and Chance, M. R. (1993) *J. Am. Chem. Soc. 115*, 2146−2150.
15. Wirt, M. D., Wu, J.-J., Scheuring, E. M., Kumar, M., Ragsdale, S. W., and Chance, M. R. (1995) *Biochemistry 34*, 5269−5273.
16. Hilhorst, E., Iskander, A. S., Chen, T. B. R. A., and Pandit, U. (1993) *Tetrahedron Lett. 34*, 4257−4260.
17. Hilhorst, E., Iskander, A. S., Chen, T. B. R. A., and Pandit, U. (1994) *Tetrahedron 50*, 8863−8870.
18. Zhao, S. Y., and Ragsdale, S. W. (1996) *Biochemistry 35*, 2476−2481.
19. Kallen, R. G., and Jencks, W. P. (1966) *J. Biol. Chem. 241*, 5845−5850.
20. Knowles, J. (1976) *CRC Crit. Rev. Biochem.*, 165−173.
21. Gupta, V. S., and Huennekens, F. M. (1967) *Arch. Biochem. Biophys. 114*, 712−718.
22. Temple, C., Bennett, L. L., Rose, J. D., Elliott, R. D., Montgomery, J. A., and Mangum, J. H. (1982) *J. Med. Chem. 25*, 161−166.
23. Elliott, J. I., and Brewer, J. M. (1978) *Arch. Biochem. Biophys. 190*, 351−357.
24. Jarrett, J. T., Choi, C. Y., and Matthews, R. G. (1997) *Biochemistry 36*, 15739−15748.
25. Fitzpatrick, P. F., and Massey, V. (1982) *J. Biol. Chem. 257*, 9958−9962.
26. Hiromi, K. (1979) in *Kinetics of fast enzyme reactions*. Halstead Press, New York.
27. El Kasmi, A., Rajasekharan, S., and Ragsdale, S. W. (1994) *Biochemistry 33*, 11217−11224.
28. Schowen, R. L. (1978) in *Transition states of biochemical processes* (Gandour, R. D., and Schowen, R. L., Eds.) Plenum Press, New York.
29. Ludwig, M. L., and Matthews, R. G. (1997) *Annu. Rev. Biochem. 66*, 269−313.
30. Seravalli, J., Zhao, S. Y., and Ragsdale, S. W. (1999) *Biochemistry 38*, 5728−5735.
31. Taylor, R. T., Norrell, S. A., and Hanna, M. L. (1972) *Arch. Biochem. Biophys. 148*, 366−381.

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