Mechanistic Studies of the Methyltransferase from *Clostridium thermoaceticum*: Origin of the pH Dependence of the Methyl Group Transfer from Methyltetrahydrofolate to the Corrinoid/Iron−Sulfur Protein†

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ABSTRACT: A methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase (MeTr) from *Clostridium thermoaceticum* catalyzes the transfer of the N^5 methyl group from (6*S*)-methyltetrahydrofolate (CH_3-H_4folate) to the cobalt center of a corrinoid/iron−sulfur protein (C/Fe-SP). The methylcobamide product is the first in a series of enzyme-bound organometallic intermediates in the acetyl-CoA pathway of anaerobic CO_2 fixation. The mechanisms of the forward and reverse reactions with CH_3-H_4folate and either the C/Fe-SP or vitamin B_{12} as substrates were studied by steady-state and pre-steady-state kinetics. This ability to effectively utilize free cobalamin as well as the C/Fe-SP in the transmethylation appears to explain why [^{14}C]methylcobyric acid was found as a product of labeling *C. thermoaceticum* cells with $^{14}CO_2$ [Ljungdahl, L. G., Irion, E., & Wood, H. G. (1965) *Biochemistry 4*, 2771−2780]. Stopped-flow experiments indicate that the Co(I)-C/Fe-SP performs a direct S_N2 displacement of the methyl group of CH_3-H_4folate to form H_4folate and methyl-Co(III). The pre-steady-state rate constants in the forward and reverse reactions increased as the pH was lowered ($pK_a \sim 5.5$). Similar pH profiles were obtained by steady-state kinetics. The k_{cat}/K_m values for the C/Fe-SP and CH_3-H_4folate in the forward direction and for the methylated C/Fe-SP and H_4folate in the reverse direction increased as the pH was lowered ($pK_a \sim 5.3$). A different pH profile was obtained with free cobalamin as the substrate; the k_{cat}/K_m for CH_3-H_4folate and cobalamin (forward reaction) increased ($pK_a \sim 7.0$) and the k_{cat}/K_m for H_4folate and methylcobalamin (reverse reaction) decreased ($pK_a \sim 5.3$) as the pH was lowered. Thus, in the methylation of B_{12}, the rate-limiting step is different from that in the methylation of the C/Fe-SP. The kinetic pK_a values closely matched the pK_a for the N^5 group of CH_3-H_4folate ($pK_a = 5.1$); however, the pH dependence of methyl transfer appears to result from ionization of a group on MeTr, not substrate. The ionization on MeTr results in a conformational change that is currently being studied.

Clostridium thermoaceticum and other anaerobic organisms can obtain all their cell carbon and energy from CO or H_2/CO_2 by the acetyl-CoA pathway (Ragsdale, 1991). CO_2 is reduced to formate and converted to (6*S*)-methyltetrahydrofolate (CH_3-H_4folate) by the action of formate dehydrogenase and a series of H_4folate-dependent enzymes. Conversion of the methyl group of CH_3-H_4folate and CO to the methyl and carbonyl groups of acetyl-CoA involves enzyme-bound organometallic intermediates. The first in this series of unique reactions is catalyzed by a methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase (MeTr).[1] In this reaction, the N^5 methyl group of CH_3-H_4folate is transferred to the cobalt center of a corrinoid/iron−sulfur protein (C/Fe-SP) (eq 1). The mechanism of this reaction is the focus of this paper. After formation of methylcobamide, the methyl group is transferred to carbon monoxide dehydrogenase (CODH) to form a methylnickel intermediate (Kumar *et al.*, 1995) that combines with a Fe−CO species (Qiu *et al.*, 1994, 1995) and CoA to form acetyl-CoA.

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

MeTr has been purified to homogeneity (Drake *et al.*, 1981) and crystallized (Doukov *et al.*, 1995). It is a homodimer with a subunit molecular mass of 28.64 kDa (Roberts *et al.*, 1994). Lacking metals or cofactors, MeTr is oxygen stable (Roberts *et al.*, 1994). The MeTr gene has been cloned, sequenced, and actively expressed in *Escherichia coli* (Roberts *et al.*, 1994). A region in the sequence of MeTr shares significant homology with a region in the *E. coli* cobalamin-dependent methionine synthase sequence that has been suggested to represent the H_4folate binding domain (Roberts *et al.*, 1994).

The natural substrate for MeTr, the C/Fe-SP, is an 88 kDa heterodimeric protein with 55 and 33 kDa subunits (Hu *et al.*, 1984; Ragsdale *et al.*, 1987). The small subunit binds 5′-methoxybenzimidazolylcobamide, and the large subunit contains a $[4Fe\text{-}4S]^{2+/1+}$ cluster (Lu *et al.*, 1993). During

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[1] Abbreviations: MeTr, methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase; C/Fe-SP, corrinoid/iron−sulfur protein; CODH, carbon monoxide dehydrogenase; CH_3-H_4folate, methyltetrahydrofolate; FdII, ferredoxin II; PCA, protocatechuic acid; PCD, protocatechuate dioxygenase; CH_3-B_{12}, methylcobalamin; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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catalysis, the C/Fe-SP cycles between the methyl-Co(III) and the Co(I) states. Whenever Co(I) escapes from the catalytic cycle by oxidation to Co(II), reductive activation is required to regenerate the active Co(I) state (Harder *et al.*, 1989). The Co(II/I) couple of the cobamide bound to the C/Fe-SP has a midpoint redox potential (E_o') of −523 mV vs the standard hydrogen electrode (SHE) (Harder *et al.*, 1989). Although this is a low-potential couple, it is ~90 mV more positive than the E_o' for free cobalamin. The C/Fe-SP facilitates the reduction by removing the benzimidazole base of the cobamide from coordination with cobalt, maintaining Co(II) in a four-coordinate state, and increasing the effective charge on cobalt relative to the free cofactor (Ragsdale *et al.*, 1987; Harder *et al.*, 1989; Wirt *et al.*, 1993, 1995).

The MeTr reaction is similar to the first half-reaction of methionine synthase (Banerjee & Matthews, 1990) in which the N^5 methyl group of CH_3-H_4folate is transferred to bound cob(I)alamin, forming an intermediate methylcob(III)amide species. The methyl group is subsequently transferred to homocysteine to form methionine. The work described here demonstrates that, like methionine synthase, MeTr uses a highly nucleophilic Co(I) species to remove the methyl group of CH_3-H_4folate. Since removal of a methyl group from a tertiary amine is difficult, it has been suggested that the N^5 group of CH_3-H_4folate is activated by protonation or oxidation (Matthews *et al.*, 1990). Chemical-modeling studies indicate that quaternization of N^5 by protonation or electrophilic coordination can activate CH_3-H_4folate (Hilhorst *et al.*, 1993, 1994). The studies described here demonstrate that the MeTr reaction is strongly pH-dependent. Although protonation of CH_3-H_4folate may be important in the enzymatic mechanism, another step in the enzymatic mechanism is responsible for the pH dependence of the MeTr reaction. This step involves ionization of a residue on MeTr resulting in a conformational change that facilitates substrate binding. Studies reported here indicate that it is the promiscuity of MeTr with enzyme-bound and free cobamides that may explain the observation three decades ago that [$^{14}CH_3$]methylcobyric acid is formed when cells of *C. thermoaceticum* are labeled with $^{14}CO_2$.

MATERIALS AND METHODS

Materials. (6*S*)-CH_3-H_4folate was a generous gift from SAPEC S.A. in Switzerland. (6*S*,6*R*)-H_4folate was purchased from Dr. B. Schircks Laboratories in Switzerland. $Ti^{III}Cl_3$ was obtained from Aldrich. Hydroxocobalamin ($B_{12}OH$), methylcobalamin (CH_3-B_{12}), and protocatechuic acid (PCA) were bought from Sigma. Protocatechuate dioxygenase (PCD) was a generous gift from Dr. David P. Ballou (The University of Michigan) and Dr. John Lipscomb (The University of Minnesota). Other reagents were of the highest purity available and were used without further purification.

Enzyme Preparation. C. thermoaceticum was grown as described (Andreesen *et al.*, 1973). All proteins were purified at 16 °C under strictly anaerobic conditions in either a Vacuum Atmospheres or a Coy Laboratory Products chamber with O_2 level below 10 ppm. The C/Fe-SP (Ragsdale *et al.*, 1987), MeTr (Hu *et al.*, 1984), ferredoxin II (FdII) (Elliott & Ljungdahl, 1982), and CODH (Ragsdale *et al.*, 1983) were purified to homogeneity as described. C/Fe-SP stock solutions were desalted using an Amicon Microcon concentrator (Model 30) by washing with three volumes of 1 mM Tris-HCl (pH 7.6). MeTr was desalted with a Model 3 Microcon using 10 mM Tris-maleate buffer (pH 5.8). The concentration of protein was determined by the Rose Bengal method (Elliott & Brewer, 1978).

Pre-Steady-State Kinetic Experiments. Pre-steady-state kinetic experiments were performed at 25 °C on a DX.17MV sequential stopped-flow ASVD spectrofluorimeter from Applied Photophysics (England). The temperature of the mixing chamber was controlled by a circulating water bath. The water bath and the mixing chamber were continually bubbled with nitrogen gas. The syringes, tubing, and mixing chamber of the stopped-flow apparatus were deoxygenated by filling the drive syringes with a solution containing 400 μM PCA and 0.2 unit of PCD in 50 mM KP_i buffer (pH 7.6), mixing the solutions, and waiting for at least 4 h before the experiments were conducted. The C/Fe-SP was reduced either by incubation with 0.1−0.2 μM CODH and 0.2 μM FdII in a CO-saturated buffer containing 1−2 mM Tris-HCl (pH 7.6) or by addition of a 10-fold excess of titanium(III) citrate. Typically, the results from three shots, each containing 1000 data points, were averaged and fitted to eq 2 where C is the amplitude, k is the rate constant, and b is the offset value to account for a non-zero base line. Other details are described in the figure legends.

$$A = Ce^{-kt} + b \tag{2}$$

The ionic strength of the MeTr reaction mixture was varied by addition of aliquots of a 6 M NaCl stock solution to the MeTr solution. When Tris-maleate was the buffer, the data were corrected for variation in the ionic strength as the pH changed by eq 3. The concentrations of maleate$^-$ and maleate^{2-} at each pH were determined by assuming pK_a values of 1.83 (pK_{a1}) and 6.07 (pK_{a2}). The concentration of $TrisH^+$ was determined assuming a pK_a of 8.1. The concentration of Na^+ was equal to the amount of NaOH added. At each pH, the fractional change in rate due to the change in ionic strength was calculated and the observed rates were adjusted by the calculated increment.

$$I = 0.5([\text{maleate}^-] + 2^2[\text{maleate}^{2-}] + [\text{TrisH}^+] + [\text{Na}^+]) \tag{3}$$

Solvent Isotope Effect Studies. For the solvent deuterium isotope effect studies, MeTr and the C/Fe-SP were extensively washed with 1 mM Tris-HCl and 0.1 M NaCl (pH 7.6) in D_2O or H_2O. CH_3-H_4folate was dissolved in the same buffer. Then 20 μM reduced C/Fe-SP (reduced by CO and CODH as described above) was rapidly mixed with 20 μM MeTr and 100 μM (6*S*)-CH_3-H_4folate in 50 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer in D_2O (pD = 6.0) or H_2O (pH = 5.6) at 25 °C. The pH and pD were measured by a pH meter. The pH electrode was soaked in D_2O or H_2O.

Methylation of the C/Fe-SP. The reaction was performed in 50 mM MES or potassium succinate buffer containing 2 mM dithiothreitol at 25 °C either on a modified Cary-14 spectrophotometer (On-Line Instrument Systems, Inc.) or on the stopped-flow instrument described above. All solutions were made anaerobic by bubbling with N_2 or preparing in the anaerobic chamber. The ionic strength was adjusted to 0.1 M by adding NaCl. The C/Fe-SP was reduced to the

Co(I) state as described above. The absorbance at 390 nm was followed and fitted to eq 4, where A_0, A, and A_i are the absorbance at time 0, t, and infinity, respectively. Other details are described in the figure legends.

$$A = (A_0 - A_i)e^{-kt} + A_i \quad (4)$$

Demethylation of CH_3-C/Fe-SP. The C/Fe-SP was methylated by first reducing it to the Co(I) state with a 2-fold excess of titanium(III) citrate and then adding a 6-fold excess of methyl iodide. The methylated C/Fe-SP was then purified from the reagents by concentrating and diluting the sample with 50 mM Tris-HCl (pH 7.6) three times using an Amicon Microcon concentrator (Model 30). The demethylation studies were performed on either the stopped-flow instrument or the spectrophotometer. The absorbance increases at 390 or 550 nm were followed, and the data were fitted to eq 5. Other details are described in the figure legends.

$$A = (A_i - A_0)e^{-kt} + A_0 \quad (5)$$

Methylation and Demethylation of B_{12} or CH_3-B_{12}. All reactions were performed in 50 mM MES or HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) buffer (the pH was varied as shown) at either 25 or 55 °C on the spectrophotometer. The ionic strength of the buffer was corrected to 0.1 M by adding NaCl. The absorbance at 520 nm was fitted to eqs 4 or 5 or to the integrated Michaelis–Menten equation (eq 6), in which t is the time. Other details are described in the figure legends.

$$t = \frac{A - A_0}{V_{max}} + \frac{1}{V_{max}/K_m} \ln\left(\frac{A_i - A_0}{A_i - A}\right) \quad (6)$$

Data Analysis. All fits were performed by nonlinear regression using the program Sigma-Plot (Jandel Scientific). Steady-state kinetic studies were performed under first- or zero-order conditions. For the first-order condition, the concentration of one substrate was much less than its K_m and the other substrate was in large excess. To determine the V/K, data were then fitted to eq 7 when substrate depletion was followed or eq 8 when product formation was followed.

$$A = (A_0 - A_i)\exp[(-V/K)t] + A_i \quad (7)$$

$$A = (A_i - A_0)\exp[(-V/K)t] + A_0 \quad (8)$$

For the zero-order experiments, both substrates were present in greater concentrations than their K_m values and one substrate was in large excess to drive the reaction to completion. The data were fitted to the integrated Michaelis–Menten equation (eq 6) to yield V_{max} and V_{max}/K_m.

RESULTS

Determination of the pK_a value for N^5 of (6S)-CH_3-H_4-folate

When its N^5 group is protonated, (6*S*)-CH_3-H_4folate fluoresces with an emission maximum at 360 nm (Figure 1, inset). The unprotonated form does not fluoresce. By measuring the fluorescence intensity as a function of pH, we determined the pK_a of the N^5 group of (6*S*)-CH_3-H_4folate (Figure 1) to be 5.05. This is similar to the value determined earlier by UV absorption spectroscopy, 5.0 (Roberts, 1992). It is also similar to the pK_a of the N^5 group of H_4folate (4.82) (Blakeley & Benkovic, 1984).

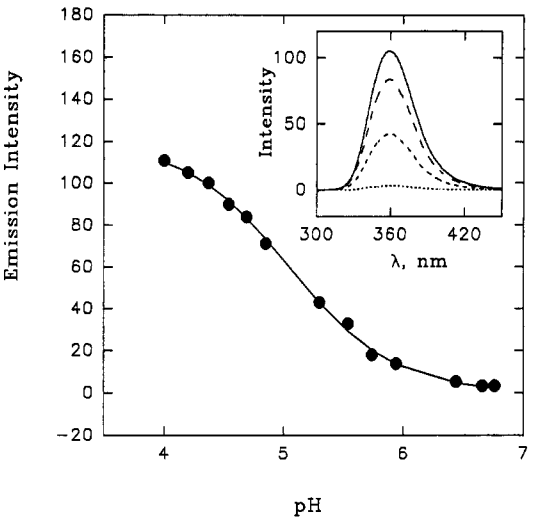

FIGURE 1: Determination of the pK_a of (6*S*)-CH_3-H_4folate. CH_3-H_4folate (0.3 μM) in 50 mM MES or acetic acid buffer was excited at 295 nm, and the emission intensity at 360 nm was measured. The ionic strength of the buffer was maintained at 0.1 M by addition of appropriate amounts of NaCl. The emission intensity at 360 nm was plotted vs pH and fitted to the equation $Y = A/(1 + 10^{pH-pK_a})$. The pK_a was determined to be 5.05 ± 0.02. Inset: emission spectrum after excitation at 295 nm.

Methylation of the C/Fe-SP

Pre-Steady-State Kinetics. The cobamide center in the C/Fe-SP can exist in the 1+, 2+, and 3+ states. Cob(I)amide is characterized by absorption peaks at 390 nm (ϵ = 30 mM^{-1} cm^{-1}) and 550 nm (ϵ = 7.5 mM^{-1} cm^{-1}), and methylcob(III)amide has an absorption peak at 450 nm (ϵ = 12.5 mM^{-1} cm^{-1}). A solution containing CH_3-H_4-folate and reduced C/Fe-SP (cob(I)amide state) was mixed rapidly with an equal concentration of MeTr. Absorption peaks at 390 and 550 nm decreased in intensity as a 450 nm peak increased (Figure 2). The apparent slight shift in the absorbance peak at 390 nm is due to the "point-by-point" method of data collection since the monochromator was advanced at 20 nm intervals for each kinetic experiment. The magnitudes of these absorption changes are consistent with 80% conversion of Co(I) to methyl-Co(III). Then the reaction was followed at 390, 450, and 550 nm (Figure 2, inset). The data collected at 390 and 450 nm fit single exponential equations with rate constants of 22 s^{-1}. By following the reaction at 550 nm, we determined the rate constant to be 15 s^{-1}. The values at 450 and 390 nm are more reliable since the absorption changes are larger than at 550 nm. These results strongly indicate that this is an S_N2-type reaction in which the methyl group of CH_3-H_4folate is displaced by cob(I)amide to generate methylcob(III)amide because the Co(I) decay rate equaled the rate of methyl-Co(III) formation and clean isosbestic points were observed on either sides of the characteristic absorption maxima.

The rate constant for C/Fe-SP methylation by CH_3-H_4-folate increased as the ionic strength was increased (Figure 3). Analysis of the data by the Debye-Huckel equation (Figure 3, inset) showed a biphasic dependence with the

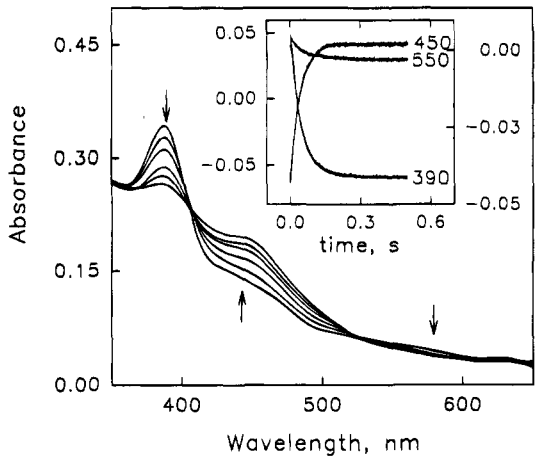

FIGURE 2: Pre-steady-state kinetics of the MeTr reaction. C/Fe-SP (15 μM) was reduced to the cob(I)amide state by incubation with CO, CODH, and ferredoxin in 1 mM Tris-HCl (pH 7.6), in a tonometer. (6*S*)-CH_3-H_4folate (100 μM) was added, and this solution was rapidly mixed with a solution containing 20 μM MeTr in 50 mM MES (pH 6.44) in the stopped-flow instrument. Separate stopped-flow experiments were performed as the wavelength was stepped from 350 to 650 nm at 20 nm intervals. The data were then collated and smoothed by a cubic spline fit by the "point-by-point" method (APS-provided software) to give plots of absorbance vs wavelength. The traces represent the spectra of the reaction after 0.05, 0.2, 0.2, 0.4, 0.65, and 2.5 s. Inset: Pre-steady-state kinetics of the MeTr reaction. The conditions were the same as above except that MeTr was in 50 mM Tris-maleate, (pH 5.8) and 100 mM NaCl. The reaction was followed at 390 nm (left-hand axis), 450 nm, and 550 nm (right-hand axis) in separate reactions. The k_{obs} was obtained by fitting the absorbance decrease and increase at 390, 450, and 550 nm to a single exponential equation (see Materials and Methods) to obtain values k_{390} = 22 ± 1 s^{-1}, k_{450} =24 ± 1 s^{-1}, and k_{550} = 15 ● 2 s^{-1}.

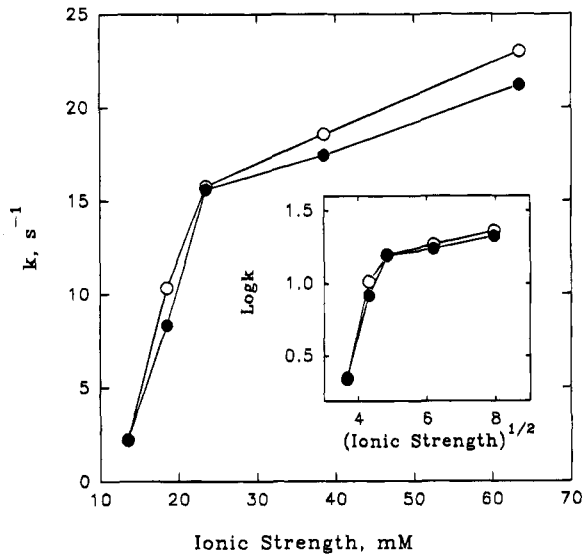

FIGURE 3: Ionic strength dependence of the methyl transfer from (6*S*)-CH_3-H_4folate to the C/Fe-SP. A solution containing 100 μM (6*S*)-CH_3-H_4folate and 15 μM reduced C/Fe-SP in 1 mM Tris-HCl buffer (pH 7.6) was mixed with 20 μM MeTr in 10 mM Tris-maleate buffer (pH 5.8) at different ionic strengths. The ionic strength was increased by increasing the concentration of NaCl in the MeTr solution. The rates of the absorbance decrease at 390 nm and increase at 450 nm were plotted against the ionic strength. Inset: data plotted according to the Debye–Huckel equation, $\log(k_{obs}) = -1/2Bz_A I^{1/2}$.

greatest sensitivity at low ionic strength. This indicates that the rate-determining step(s) at low and high ionic strengths

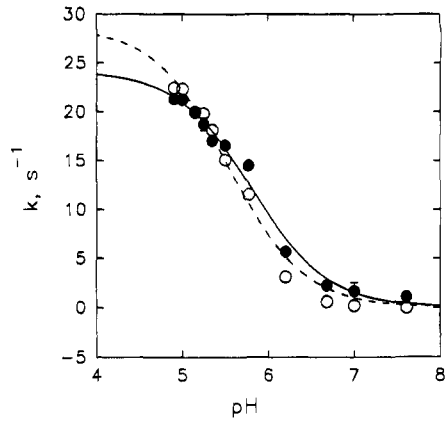

FIGURE 4: pH dependence of the methyl transfer from (6*S*)-CH_3-H_4folate to C/Fe-SP. C/Fe-SP (20 μM) was reduced by CODH, ferredoxin, and CO in 2 mM Tris-maleate buffer (pH 7.2) in a total reaction volume of 8 mL. The solution was then reacted rapidly with 100 μM (6*S*)-CH_3-H_4folate and 20 μM MeTr in 50 mM potassium succinate or Tris-maleate buffer at various pH values. The k_{obs} for the absorbance decrease at 390 nm was plotted vs pH and fitted to a single pH titration equation. The dashed line is the fitting after correction for the ionic strength. The maximum rate constants and pK_a values are given in Table 1.

may be different. We performed the rest of our experiments at ionic strengths above 25 mM.

Methylation of the C/Fe-SP by CH_3-H_4folate was studied between pH 4.9 and 7.6 (Figure 4). MeTr precipitates below its isoelectric point, which is 4.8. As the pH was lowered, the k_{obs} increased according to a pK_a of 5.8 (solid line). These studies were performed with either potassium succinate (below pH 5.2) or Tris-maleate buffer in which the ionic strength of the buffer varies with pH. Since the rate of the reaction increases as the ionic strength increases, the measured rates were corrected to the same ionic strength (0.03 M). The pK_a for the corrected data (dashed line) was 5.5.

If the pH dependence of methylation of the C/Fe-SP resulted from proton transfer from solvent to the substrate, the reaction could be subject to a solvent deuterium isotope effect. When the reaction was performed in H_2O, the rate constants were 7.0 (390 nm), 7.4 (450 nm), and 7.4 (550 nm) s^{-1} in water. The rate constants were 7.9 (390 nm), 9.1 (450 nm), and 7.1 (550 nm) s^{-1} when the reaction took place in D_2O. These results indicate that the rate constant was ~7.4 in either water or D_2O, and thus, there was no solvent isotope effect on the methylation of the C/Fe-SP.

Steady-State Kinetics. The reactions of CH_3-H_4folate with the C/Fe-SP and with vitamin B_{12} were studied by steady-state kinetics in the forward and reverse directions. In addition, the pH dependencies of these reactions were determined. The pK_a values and the maximum values of the kinetic constants are listed in Table 1.

pH Dependence of the k_{cat}/K_m for the C/Fe-SP and CH_3-H_4folate. The k_{cat}/K_m for the C/Fe-SP was determined by decreasing the C/Fe-SP concentration to 20-fold less than its K_m and 40-fold less than the CH_3-H_4folate concentration. The conversion of Co(I) to CH_3-Co(III) was followed at 390 nm ($\Delta\epsilon_{390}$ = 17 mM^{-1} cm^{-1}). The k_{cat}/K_m for the C/Fe-SP was determined between pH 4.9 and 6.6 (Figure 5, ●). The reaction went to completion at all pH values. The data were fitted to eq 9 to yield a pK_a of 5.2 and a maximum value (at low pH) of 1.6 × 10^6 M^{-1} s^{-1}.

Table 1: Kinetic Constants for the Forward and Reverse Reactions of MeTr

	forward reaction		reverse reaction		forward reaction		reverse reaction	
	C/Fe-SP	CH_3-H_4folate	CH_3-C/Fe-SP	H_4folate	$B_{12}OH$	CH_3-H_4folate	CH_3B_{12}	H_4folate
K_m, μM	60	10	>100	>200	2000	10	665	341
k_{cat}, s^{-1}	18		>0.02		1.1	1.5 ± 0.1	2.2	
max k_{cat}/K_m, $M^{-1}\ s^{-1}$ ($\times 10^{-3}$)	1600 ± 100	$10\,800 \pm 1800$	17.1 ± 0.7	5.2 ± 0.2	14.7 ± 1	450 ± 20	2.1 ± 0.7	4.4 ± 0.5
pK_a, k_{cat}/K_m pK_a, k_{cat}	5.2 ± 0.1	5.0 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	7.0 ± 0.1	7.1 ± 0.1 7.1 ± 0.1	5.6 ± 0.1	5.1 ± 0.1
max k_{obs}, s^{-1}	24 ± 1^a 29 ± 2^b		34 ± 1^a 40 ± 2^b					
pK_a	5.8 ± 0.1^a 5.5 ± 0.1^b		5.9 ± 0.1^a 5.7 ± 0.1^b					

[a] Before correction. [b] After correction.

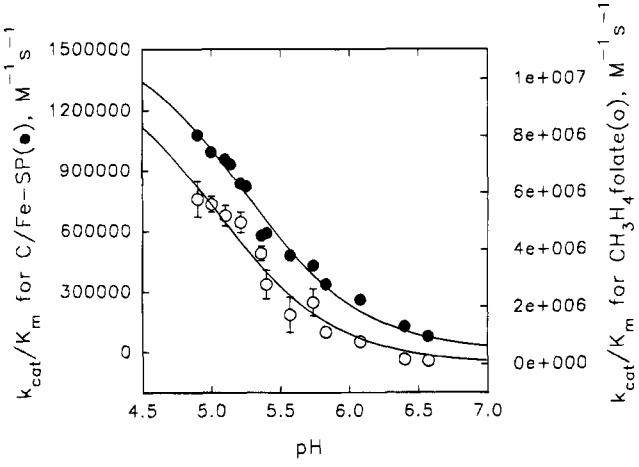

FIGURE 5: pH dependence of methylation of C/Fe-SP. For the determination of the k_{cat}/K_m of C/Fe-SP (●), the reaction mixture contained 2.9 μM C/Fe-SP, 120 μM CH_3-H_4folate, 2.1 mM titanium(III) citrate, and 54 nM MeTr in 800 μL of 50 mM MES or potassium succinate. The reaction was performed at 25 °C. The absorbance decrease at 390 nm was followed, and the data were fitted to eq 7. To obtain the pH dependence of k_{cat}/K_m of CH_3-H_4-folate (○), 46 μM C/Fe-SP in 50 mM Tris-Cl and 0.1 M NaCl was reduced by 0.4 μM CODH and CO from Co(II) to Co(I). The solution was rapidly mixed with 3 μM CH_3-H_4folate and 0.15 μM MeTr in 50 mM MES or potassium succinate buffer on the stopped-flow instrument. The reaction was performed at 25 °C in the presence of 1 mM titanium(III) citrate. The absorbance decrease at 390 nm was followed, and the data were fitted to eq 7. The limiting values of k_{cat}/K_m and the pK_as are given in Table 1.

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{max}}{1 + 10^{pH-pK_a}} \quad (9)$$

To obtain the k_{cat}/K_m for CH_3-H_4folate, the concentration of CH_3-H_4folate was decreased to 7-fold less than its K_m and 15-fold less than the concentration of C/Fe-SP. The final concentrations of CH_3-H_4folate, C/Fe-SP, and MeTr were 1.5 μM, 23 μM, and 75 nM, respectively. The pH dependence of the k_{cat}/K_m for CH_3-H_4folate was then determined (Figure 5, ○). The kinetic traces exhibited a single exponential decay at all pH values, and the amount of C/Fe-SP methylated was equivalent to the concentration of CH_3-H_4folate. Thus, all the CH_3-H_4folate present was demethylated. When the k_{cat}/K_m values were plotted vs pH and fitted to eq 9, the data paralleled that for the k_{cat}/K_m for the C/Fe-SP and the fit pK_a was 5.0 (solid line).

Demethylation of CH_3-C/Fe-SP by H_4folate

Pre-Steady-State Kinetics. Methylation of the C/Fe-SP by CH_3-H_4folate was earlier described as irreversible (Hu *et al.*, 1984). However, Figure 6 shows clearly that the reaction is reversible. The absorbance at 390 nm increased and the 450 nm absorbance decreased with clean isosbestic points, indicating that CH_3-Co(III) was converted directly to Co(I) and that the reaction occurs by an S_N2 displacement of the methyl group by H_4folate. This supports the direct displacement mechanism proposed above for the forward reaction by the principle of microscopic reversibility.

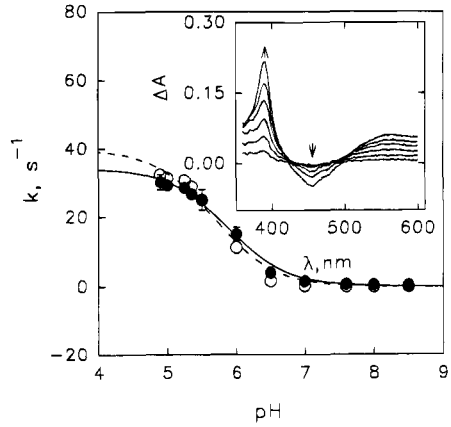

FIGURE 6: Pre-steady-state kinetics of the demethylation reaction. CH_3-C/Fe-SP (20 μM) in 2 mM Tris-maleate buffer (pH 7.2) reacted rapidly with 100 μM H_4folate and 20 μM MeTr in 100 mM Tris-maleate or potassium succinate buffer on the stopped-flow machine. The reaction was studied at 25 °C. The rates of absorbance decrease at 390 nm were plotted against pH, and the pK_a was 5.9 ± 0.1 (solid line). After correction of the data for the ionic strength variation as a function of pH (dashed line), the pK_a was determined to be 5.7 ± 0.1 with a limiting value of 40 ± 2. Inset: demethylation of CH_3-C/Fe-SP by H_4folate. CH_3-C/Fe-SP (15 μM) reacted with 840 μM H_4folate and 4.6 μM MeTr in 150 μL of 50 mM HEPES buffer (pH 7.93) in the presence of 2 mM titanium-(III) citrate at 25 °C. Spectra were collected as the reaction proceeded.

The reaction was then followed at 390, 450, and 550 nm. A solution containing 100 μM H_4folate and 20 μM MeTr was rapidly mixed with 20 μM CH_3-C/Fe-SP at different pH values. The rates of absorbance increase at 390 nm (Co(I) formation) and decrease at 450 nm (methyl-Co(III) depletion) were identical, indicating that CH_3-Co(III) was converted to Co(I) without the intermediacy of Co(II). The pK_a was determined to be 5.9 (Figure 6). After the data were corrected for the ionic strength of the buffer, the pK_a was 5.7, closely matching the pK_a for the forward reaction.

Steady-State Kinetics. pH Dependence of the k_{cat}/K_m for CH_3-C/Fe-SP and H_4folate. Preliminary data indicated that the K_m values for CH_3-C/Fe-SP and H_4folate were above 100 and 200 μM, respectively, at pH 7.6 and that the k_{cat} was

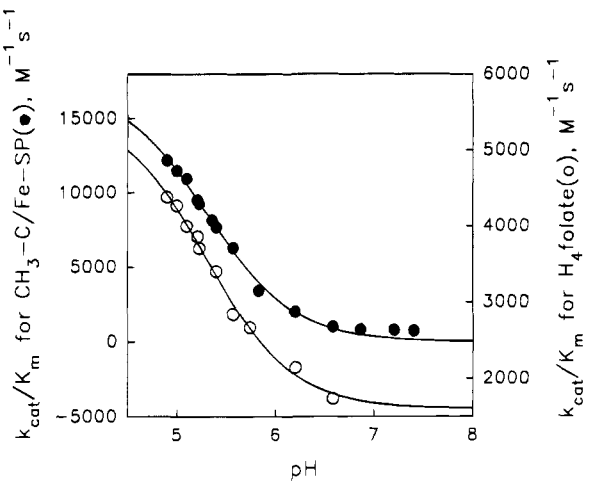

FIGURE 7: pH dependence of demethylation of CH_3-C/Fe-SP. To obtain the k_{cat}/K_m for CH_3-C/Fe-SP (●), the reaction mixture contained 17 μM CH_3-C/Fe-SP, 492 μM H_4folate, 0.16 μM MeTr, and 3 mM titanium(III) citrate in 500 μL of 50 mM MES or potassium succinate buffer. The reaction was studied on the spectrophotometer at 55 °C. The absorbance increase at 390 nm was followed, and the calculated values of k_{cat}/K_m were plotted against the pH. To obtain the k_{cat}/K_m for H_4folate (○), the reaction mixture contained 55 μM CH_3-C/Fe-SP, 10 μM H_4folate, 0.8 μM MeTr, and 3 mM titanium (III) citrate in 500 μL of 50 mM MES or potassium succinate buffer. The reaction was studied at 35 °C on the spectrophotometer. The absorbance increase at 550 nm was followed, and the rate was plotted against pH. The limiting values of k_{cat}/K_m and the pK_as are given in Table 1.

greater than 0.02 s^{-1} at 25 °C. The pH dependence of the k_{cat}/K_m for CH_3-C/Fe-SP (Figure 7, ●) was obtained by lowering the concentration of CH_3-C/Fe-SP to ~6-fold less than its K_m and 14-fold less than the concentration of (6*S*)-H_4folate. As with the forward reaction, the k_{cat}/K_m increased as the pH was lowered. On the basis of the absorption change, all the C/Fe-SP present was demethylated. Plotting the data versus pH and fitting to eq 9 yielded a pK_a of 5.3.

The pH dependence of the k_{cat}/K_m for H_4folate (Figure 7, ○) was obtained by decreasing the concentration of H_4folate to 20-fold less than its K_m and 11-fold less than the concentration of CH_3-C/Fe-SP. From the magnitude of the absorption change, all the H_4folate present was found to be methylated. The k_{cat}/K_m increased as the pH decreased with a pK_a of 5.3.

Methylation of B_{12}

pH Dependence of the k_{cat}/K_m for Hydroxocobalamin and (6S)-CH_3-H_4folate. MeTr can use the C/Fe-SP or free corrinoids as substrate to reversibly transfer the methyl group of (6*S*)-CH_3-H_4folate to Co(I)-B_{12}. The reaction uses a ternary complex mechanism with K_m values of 2 mM for hydroxocobalamin and 10 μM for CH_3-H_4folate at pH 7.6 (Roberts, 1992). The k_{cat} is 1.1 s^{-1} at 25 °C. For obtaining the k_{cat}/K_m for hydroxocobalamin, the concentration of B_{12} was ~260-fold less than its K_m and ~100-fold less than that of CH_3-H_4folate. The increase in absorbance at 520 nm due to formation of CH_3-B_{12} was followed, and the data were fitted to eq 8. As shown in Figure 8, the reaction rate increased as the pH was lowered according to a pK_a of 7.0.

In order to determine the k_{cat}/K_m for (6*S*)-CH_3-H_4folate, the reaction was performed with 20-fold less CH_3-H_4folate than hydroxocobalamin. The absorbance at 520 nm was followed, and the k_{cat}/K_m and k_{cat} were calculated by fitting

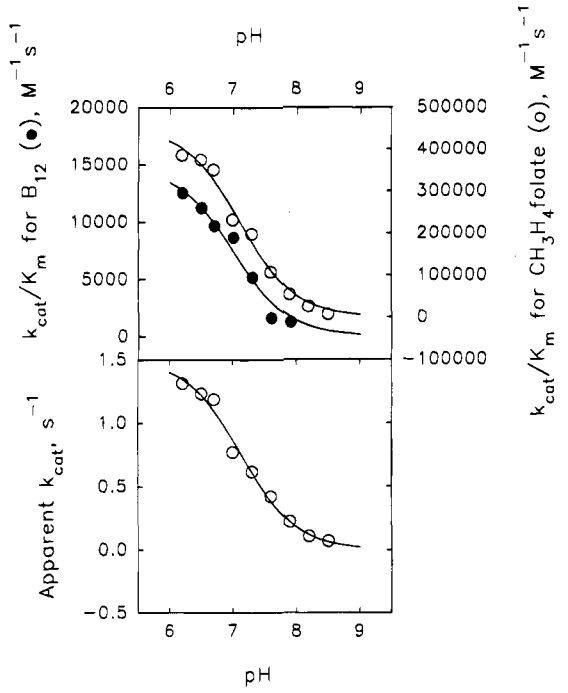

FIGURE 8: pH dependence of methylation of hydroxocobalamin. To obtain the k_{cat}/K_m for B_{12}OH (●), 7.5 μM B_{12}OH was reduced by 0.5−2 mM titanium (III) citrate from Co(II) to Co(I) and reacted with 780 μM CH_3-H_4folate and 0.37 μM MeTr in 800 μL of 50 mM HEPES or MES buffer. The reaction was performed at 55 °C on the spectrophotometer. The absorbance increase at 520 nm was followed and the rate was plotted against the pH. To obtain the k_{cat}/K_m for (6*S*)-CH_3-H_4folate (top, ○) and apparent k_{cat} (bottom), 400 μM B_{12}OH was reduced by 1−4 mM titanium(III) citrate from Co(III) to Co(I) and reacted with 20 μM (6*S*)-CH_3-H_4folate and 0.35 μM MeTr in 800 μL of 50 mM HEPES or MES buffer at 25 °C. The absorbance increase at 520 nm was followed, and the data were fitted to the integrated Michaelis−Menten equation. The limiting values of k_{cat}/K_m and the pK_as are given in Table 1.

the data to eq 6. The reaction rate increased as the pH was lowered (Figure 8). The fit line is based on pK_a values of 7.1 for both k_{cat}/K_m and k_{cat}.

Demethylation of CH_3-B_{12}

pH Dependence of k_{cat}/K_m for CH_3-B_{12} and H_4folate. Earlier work indicated that methylation of H_4folate by CH_3-B_{12} follows a ternary complex mechanism with K_m values of 341 and 665 μM for H_4folate and CH_3-B_{12}, respectively, and a k_{cat} of 2.2 s^{-1} at 55 °C and pH 6.8 (Roberts, 1992). The conditions for obtaining the pH dependence of k_{cat}/K_m for CH_3-B_{12} included lowering the concentration of CH_3-B_{12} to ~50-fold below its K_m and 40-fold less than that of H_4folate. The reaction was followed at 520 nm. All the CH_3-B_{12} present was demethylated on the basis of the absorbance change. The rate increased as the pH was increased according to a pK_a of 5.6 (Figure 9, ●).

The pH dependence of the k_{cat}/K_m for H_4folate was obtained by lowering the concentration of H_4folate to 14-fold less than its K_m and 42-fold less than that of CH_3-B_{12} (Figure 9, ○). The absorbance at 520 was followed. On the basis of the magnitude of the absorbance change, all theH_4folate present was methylated. The rate increased as the pH was increased according to a pK_a of 5.1.

DISCUSSION

Steady-state and pre-steady-state kinetic studies were performed to better understand how MeTr catalyzes the

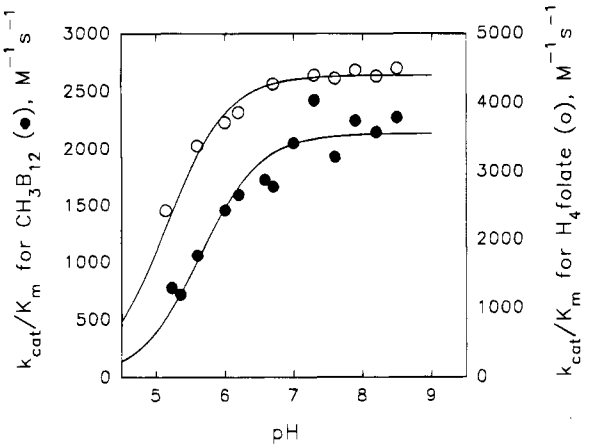

FIGURE 9: pH dependence of demethylation of CH_3-B_{12}. To obtain the k_{cat}/K_m for CH_3-B_{12} (●), the reaction mixture contained 12.5 μM CH_3-B_{12}, 1 mM H_4folate, and 0.74 μM MeTr in 800 μL of 50 mM MES or HEPES buffer. The reaction was performed at 55 °C, and the absorbance decrease at 520 was followed. The reaction went to completion, and the data were fitted to eq 7. The calculated k_{cat}/K_m were values plotted against the pH. To obtain the k_{cat}/K_m for H_4folate (○), the reaction mixture contained 24 μM H_4folate, 500 μM CH_3-B_{12}, and 1.2 μM MeTr in 500 μL of 50 mM MES or HEPES buffer. The absorbance decrease at 520 nm was followed at 55 °C. The data were fitted to eq 7, and the calculated k_{cat}/K_m values were plotted against pH. The limiting values of k_{cat}/K_m and the pK_as are given in Table 1.

methylation of the C/Fe-SP by CH_3-H_4folate. Our studies indicated that the N^5 methyl group of CH_3-H_4folate is removed in an S_N2-type displacement reaction by a Co(I) nucleophile on the C/Fe-SP. The same mechanism appears to occur when the free cofactor, B_{12}, is the substrate. First, the rate of decay of Co(I) was equivalent to the rate at which CH_3-C/Fe-SP is formed. Second, when the spectra were collected during the reaction, clean isosbestic points were observed. Third, for the reverse reaction, the rate of methyl-Co(III) decay equaled the rate of Co(I) formation. Fourth, time-resolved spectra during the reverse reaction exhibited clean isosbestic points. Thus, in the reverse reaction, H_4-folate appears to perform a nucleophilic attack on methyl-Co(III). Fifth, an S_N2 reaction would be consistent with evidence favoring a mechanism in which MeTr, CH_3-H_4-folate, and the C/Fe-SP form a ternary complex (Roberts, 1992). Sixth, an S_N2-type mechanism would conform with that of methionine synthase in which the methyl group of CH_3-H_4folate is removed by Co(I) by a direct displacement (Banerjee *et al.*, 1990).

Although it was earlier thought that methylation of the C/Fe-SP was irreversible (Hu *et al.*, 1984), these studies demonstrated conclusively that the MeTr-catalyzed reaction is reversible with both B_{12} and the C/Fe-SP as substrates. Perhaps the reason the reverse reaction was not observed before is that the K_m for H_4folate is over 20-fold higher than for CH_3-H_4folate.

Mechanistic information can be gained by comparing enzyme-bound and free cobamides as substrates.[2] Binding of H_4folate or CH_3-H_4folate to MeTr apparently is unaffected by whether the cobamide is free or protein-bound since the K_m values for the folate derivatives are similar whether B_{12} or cobamide is used. Additionally and surprisingly, in the reverse reaction, MeTr poorly discriminates between the cobamide and the protein-bound cobamide. The k_{cat}/K_m for the methylated C/Fe-SP is only 8-fold higher than that for

Table 2[a]

pH	[protonated CH_3-H_4folate], μM	total [CH_3-H_4folate], μM	k_{obs}, min^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
5.74	18.5	120	2.45 ± 0.1	$(7.6 \pm 0.3) \times 10^5$
5.74	50.4	325	2.24 ± 0.1	$(6.9 \pm 0.3) \times 10^5$
6.57	3.2	120	0.47 ± 0.02	$(1.45 \pm 0.06) \times 10^5$
6.57	50.4	1908	0.36	1.1×10^5

[a] In these studies, the concentration of C/Fe-SP was 2.9 μM.

CH_3-B_{12}. For the forward reaction, the k_{cat}/K_m for the C/Fe-SP was ~100-fold greater than that for B_{12}, with ~1 order of magnitude ascribed each to changes in k_{cat} and binding of the cobamide.[3] Although the C/Fe-SP is a significantly better methyl group acceptor than B_{12}, the levels of free corrinoids in *C. thermoaceticum* can approach 300−700 μM, approximately 10-fold higher than the concentration of the C/Fe-SP. Therefore, the in vivo rate of methylation of B_{12} and probably other free cobamides would be only ~7−17-fold slower than that of the C/Fe-SP.[4] The promiscuity of MeTr with different corrinoids may explain why both [^{14}C]-methylcobyric acid and [^{14}C]methylbenzimidazolylcobamide (the cofactor in the C/Fe-SP) were detected when cells of *C. thermoaceticum* were labeled with $^{14}CO_2$ (Ljungdahl *et al.*, 1965).

Geometric and electronic factors are thought to influence the changes in the kinetics with the C/Fe-SP relative to free B_{12}. The major differences between the C/Fe-SP and B_{12} are in the values of k_{cat} and the K_m for the cobamide. It has been shown that the C/Fe-SP profoundly alters the electronic properties of cobalt. This is reflected in making Co(II) ~60-fold easier to reduce relative to the free cofactor (Harder *et al.*, 1989) and reducing the nuclear charge on cobalt in all three oxidation states (Wirt *et al.*, 1995). Strategies for accomplishing these effects include imposing a four-coordinate geometry in the Co(II) and Co(I) states (Wirt *et al.*, 1993) and removing the benzimidazole base from coordination in all three (Co(I), Co(II), and methyl-Co(III)) states.[5] The mechanistic consequences of these changes in coordination geometry have been discussed (Harder *et al.*,

[2] B_{12} has a methyl group at both the 5 and 6 positions of the benzimidazole base, whereas the cobamide on the C/Fe-SP contains a methoxy substitution at the 5 position and no group at C^6 (Ragsdale *et al.*, 1987). Earlier reconstitution studies demonstrated that MeTr has the same activity whether the C/Fe-SP contains B_{12} or the natural cobamide (Lu *et al.*, 1993). Therefore, the differences reflect changes due to the presence of the C/Fe-SP, not minor differences in decoration of the benzimidazole between B_{12} and the natural cobamide.

[3] There is a 20-fold difference in the k_{cat}/K_m for CH_3-H_4folate when the protein and B_{12} are compared, and the K_m values are nearly identical. The k_{cat}/K_m for the C/Fe-SP is 100-fold greater than the value for B_{12}, whereas the k_{cat} is 16-fold greater and the K_m is ~30-fold smaller.

[4] The level of free corrinoids is approximately 300−700 nmol mL^{-1} of frozen cells (Ljungdahl *et al.*, 1966). The C/Fe-SP represents approximately 3% of cell protein (unpublished results). Given that the protein concentration in the growing bacterial cell is about 0.16 g g^{-1} wet weight of the cell (Ingraham *et al.*, 1983) and the molecular mass of the C/Fe-SP is 88 kDa (Hu *et al.*, 1984), the concentration of the C/Fe-SP in the cell is estimated to be approximately 55 μM. The electronic effects described in the paragraph would tend to favor cobyric acid over the complete corrinoids in solution because it does not contain the lower axial ligand and would be expected to be more reactive.

[5] Although the four-coordinate state observed for the C/Fe-SP is unique, base-off geometry is becoming a rule rather than an exception as has been shown for methionine synthase (Drennan *et al.*, 1994), methylmalonyl-CoA mutase (Padmakumar *et al.*, 1995), and a protein from *Sporomusa* (Stupperich *et al.*, 1990). In these cases, a lower axial histidine ligand from the protein is present.

Table 3[a]

pH	[protonated CH_3-H_4-folate], μM	total [CH_3-H_4-folate], μM	k, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}	amplitude
5.83	0.39	3	0.13 ± 0.005	$(1.73 \pm 0.07) \times 10^6$	0.053 ± 0.003
5.83	1.29	10	0.047 ± 0.0008	$(6.3 \pm 0.1) \times 10^5$	0.12 ± 0.0015
6.4	0.11	3	0.022 ± 0.004	$(2.9 \pm 0.5) \times 10^5$	0.044 ± 0.007
6.4	1.26	33	0.016 ± 0.0003	$(2.13 \pm 0.04) \times 10^5$	0.15 ± 0.003

[a] In these studies, the concentration of C/Fe-SP was 46 μM.

1989; Wirt *et al.*, 1995). Basically, these geometric changes stabilize Co(I) and prevent the methyl group of methyl-Co(III) from undergoing disadvantageous homolysis.

Why does MeTr bind the C/Fe-SP so weakly? A K_m value of 60 μM is likely to reflect a relatively weak binding constant. This can be understood by considering the role of the C/Fe-SP. This protein must interact with MeTr, undergo methylation, and then dissociate from MeTr to interact with CODH where the final steps of acetyl-CoA synthesis occur. Therefore, if the association between the C/Fe-SP and MeTr were too strong, the C/Fe-SP would be unavailable for delivery of the methyl group to CODH for the final steps in acetyl-CoA synthesis.

Since it is difficult to remove the methyl group chemically from the tertiary amine, one proposed mechanism to facilitate this reaction would be to protonate or oxidize the N^5 group of CH_3-H_4folate (Matthews *et al.*, 1990). This would make the methyl group more electrophilic. The kinetic pK_a values reasonably matched the pK_a value for the N^5 group of CH_3-H_4folate in solution. However, these pH dependencies could be attributed to ionization of either the substrate or the free enzyme. Accordingly, we recently demonstrated that MeTr undergoes a conformational change with a pK_a similar to the kinetic pK_a values (manuscript submitted for publication). Several experiments indicate that the pH dependence of the methyl transfer reaction is controlled primarily by ionization of the enzyme and not by the protonation state of CH_3-H_4-folate. First, if protonation of the substrate was rate determining, the k_{cat}/K_m for the C/Fe-SP would be expected to be proportional to the concentration of CH_3-H_4folate. However, when the concentration of protonated CH_3-H_4folate was varied by 3-fold at pH 5.7 and 16-fold at pH 6.6, there was no effect (Tables 2 and 3). Decreasing the pH by 0.7 unit increased the k_{cat}/K_m for the C/Fe-SP ~6-fold. Second, no significant solvent deuterium isotope effect was observed for methylation of the C/Fe-SP. A rate-limiting protonation of substrate would be expected to exhibit a significant solvent isotope effect. Third, the k_{cat}/K_m values for CH_3-H_4folate and for the C/Fe-SP follow similar pH profiles. This would be consistent with a rate-controlling enzyme ionization. Fourth, both forward and reverse reactions were accelerated by decreasing the pH and had almost identical pK_a values. If substrate ionization were responsible for the kinetic pK_a of the forward reaction, then the reverse reaction would be expected to be activated by deprotonation. The protonation state of enzyme-bound CH_3-H_4folate will have to be addressed by methods other than kinetics. Although it is likely that this proton transfer occurs, it is not responsible for the pH dependence of the MeTr reaction and could be much faster than another pH-dependent step in the mechanism. The pH dependence of the kinetics of the MeTr reaction is likely to be a pH-linked conformational change in MeTr that is the subject of current investigation.

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