A Conformational Change in the Methyltransferase from *Clostridium thermoaceticum* Facilitates the Methyl Transfer from (*6S*)-Methyltetrahydrofolate to the Corrinoid/Iron−Sulfur Protein in the Acetyl-CoA Pathway†

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ABSTRACT: The methyltetrahydrofolate:corrinoid/iron−sulfur protein methyltransferase (MeTr) from *Clostridium thermoaceticum* catalyzes the methylation of a corrinoid/iron−sulfur protein (C/Fe-SP) by the N^5 methyl group of (*6S*)-methyltetrahydrofolate (CH_3-H_4folate). This is an important reaction in the reductive acetyl-CoA pathway. The forward and reverse reactions of MeTr have a pH dependence that appears to reflect protonation of a group on the protein [Zhao, S., Roberts, D. L., & Ragsdale, S. W. (1995) *Biochemistry 34*, 15075−15083]. In the work reported here, fluorescence and rapid reaction kinetics were used to demonstrate that this protonation elicits a rate-limiting conformational change. As the pH was lowered, the emission maximum for intrinsic tryptophan fluorescence underwent a red shift (pK_a = 5.4) and the emission intensity increased (pK_a = 5.1). The extrinsic fluorescence probe, 4,4′-bis-1-phenylamino-8-napthalenesulfonate (bis-ANS) was used to report on the conformational change. The bis-ANS fluorescence was strongly enhanced upon binding MeTr. As the pH was decreased, the fluorescence was further enhanced and the emission maximum underwent a 14 nm blue shift (pK_a = 5.0). By stopped-flow fluorescence studies, it was shown that these fluorescence changes occur at rates similar to the k_{cat} for the MeTr reaction and thus reflect catalytically competent events. The combined results indicate that CH_3-H_4folate binds to a hydrophobic region in MeTr that includes a tryptophan residue(s). MeTr undergoes a pH-dependent conformational change that exposes this region to solvent and facilitates substrate binding.

Clostridium thermoaceticum and other anaerobic bacteria fix CO and CO_2 by the acetyl-CoA or Wood−Ljungdahl pathway (Ljungdahl, 1986; Ragsdale, 1991). This pathway converts 2 mol of CO or CO_2 to 1 mol of acetyl-CoA. In the first step of this pathway, (*6S*)-methyltetrahydrofolate (CH_3-H_4folate) is formed by a reaction sequence involving formate dehydrogenase and four H_4folate-dependent enzymes. Then, 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)[1] by a CH_3-H_4folate:C/Fe-SP methyltransferase (MeTr). The mechanism of MeTr is the focus of this manuscript. The methyl group from the methylated C/Fe−SP is condensed with CoA and CO to form acetyl-CoA through a series of organometallic intermediates by carbon monoxide dehydrogenase (CODH).

MeTr lacks chromophobic cofactors and metals and is oxygen stable (Roberts *et al.*, 1994). The gene encoding MeTr has been cloned (Roberts *et al.*, 1989) and can be expressed in an active form in *Escherichia coli* (Roberts *et al.*, 1994). The MeTr reaction is summarized in eq 1.

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

Although cobalt undergoes a formal two-electron oxidation, this reaction does not involve net redox chemistry because the methyl group is transferred as a cation. The MeTr-catalyzed reaction is reminiscent of that of methionine synthase (Banerjee & Matthews, 1990). In both proteins, methyl-cob(III)amide is formed from CH_3-H_4folate. The physiological methyl acceptor for MeTr is the cobamide center of the C/Fe-SP, an 88 kDa protein with 55 and 33 kDa subunits (Hu *et al.*, 1984; Ragsdale *et al.*, 1987). For methionine synthase, it is bound vitamin B_{12}. The sequence of MeTr (with a subunit molecular mass of 27 kDa) matched a similarly sized region in methionine synthase that was adjacent to the cobamide binding domain, suggesting that this region represents the H_4folate binding domain (Roberts *et al.*, 1994). There are also major differences between methionine synthase and MeTr. Methionine synthase requires S-adenosylmethionine as an activator and transfers the methyl group to homocysteine to form methionine. The MeTr from *C. thermoaceticum* is simpler. It does not require *S*-adenosylmethionine (Hu *et al.*, 1984), and methylcobamide is the final product. Subsequently, in a reaction that does not involve MeTr, the methyl group of methylcobamide is transferred to a nickel site on CODH (Kumar *et al.*, 1995) for the final steps in the acetyl-CoA pathway.

Removing a methyl group from a tertiary amine is a difficult reaction, even with a strong nucleophile like cob(I)amide. It has been suggested that this reaction could be facilitated by protonation or oxidation of the N^5 group of CH_3-H_4folate (Matthews *et al.*, 1990). This concept is

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[1] Abbreviations: 4,4′-bis(1-(phenylamino)-8-napthalenesulfonate), bis-ANS; carbon monoxide dehydrogenase, CODH; corrinoid/iron−sulfur protein, C/Fe-SP; methyltetrahydrofolate, CH_3-H_4folate; methyltransferase, MeTr; 2-[*N*-morpholino]ethanesulfonate, MES; protocatechuate dioxygenase, PCD; FdII, ferredoxin II from *C. thermoaceticum.*

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supported by model studies (Hilhorst *et al.*, 1993; Hilhorst *et al.*, 1994), however, so far there is no experimental evidence for such an activation of CH_3-H_4folate in an enzyme. Although the kinetic pK_a for the MeTr reaction was found to be similar to the pK_a of the N^5 group of CH_3-H_4folate, several results demonstrated that the pH dependence actually was controlled by ionization of the enzyme not the substrate (Zhao *et al.*, 1995). (i) The forward and reverse reactions had identical pH dependences, increasing as the pH was lowered. 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. (ii) The k_{cat}/K_m for the C/Fe-SP was not a function of the concentration of protonated CH_3-H_4folate. (iii) The k_{cat}/K_m for CH_3-H_4folate and for the C/Fe-SP followed similar pH profiles. (iv) No significant solvent deuterium isotope effect was observed for methylation of the C/Fe-SP, whereas a rate-limiting protonation of substrate would be expected to exhibit a significant solvent isotope effect. For the above reasons, it was proposed that protonation of a group on MeTr controlled the pH dependence of the transmethylation reaction (Zhao *et al.*, 1995). The work described here provides evidence that MeTr protonation results in a rate-limiting conformational change in MeTr. This conformational change appears to facilitate CH_3-H_4folate binding by exposing a hydrophobic region to solvent.

MATERIALS AND METHODS

Chemicals. (*6S*)-Methyl-H_4folate was a generous gift from SAPEC S. A. (Switzerland). 4,4′-Bis(1-(phenylamino)-8-napthalenesulfonate) (bis-ANS) was purchased from Molecular Probes. Protocatechuic acid (PCA) was purchased from Sigma, and 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 analytical grade from either Sigma or Aldrich 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 anaerobic chamber or a Coy Laboratory Products chamber with the O_2 level below 10 ppm. The C/Fe-SP (Ragsdale *et al.*, 1987), CODH (Ragsdale *et al.*, 1983) and ferredoxin (Elliott & Ljungdahl, 1982) were purified to homogeneity as described. The concentration of protein was determined by the Rose Bengal method (Elliott & Ljungdahl, 1982).

MeTr was purified 51-fold to apparent homogeneity essentially as described earlier (Drake *et al.*, 1981). MeTr was routinely measured during purification by Western hybridization using anti-MeTr antibody (Roberts *et al.*, 1989). The routine assay for MeTr (Hu *et al.*, 1984) followed the demethylation of methylcobalamin by H_4folate at 520 nm and 55 °C. The assay mixture (0.8 mL) contained 66 μM methylcobalamin, 300 μM H_4folate, and 50 mM Tris-maleate buffer, pH 8.5.

Fluorescence Measurements. Fluorescence data were collected with a Perkin-Elmer LS-50 luminescence spectrometer. The excitation source was a Xenon flash tube with an excitation monochrometer that filters the IR radiation and an emission monochrometer. The temperature of the cuvette was mantained at 25 °C using a circulating water bath. Anaerobic stock solutions of bis-ANS, CH_3-H_4folate, H_4-folate, and 2-[*N*-morpholino]ethanesulfonate (MES), potassium succinate, and Tris-HCl buffers were prepared and stored in glass vials closed with thick butyl rubber serum stoppers. Cuvettes containing the buffer were closed with a red rubber serum stopper, and N_2 gas was bubbled for 10 min in the buffer before an aliquot of a stock solution(s) was added by a gas-tight Hamilton syringe. The volume for all measurements was 2.5 mL. The fluorescence emission spectra of the folate derivatives, MeTr, and bis-ANS were determined at excitation wavelengths of 295, 295, and 405 nm, respectively. The protonated form of CH_3-H_4folate fluoresces at 360 nm; however, the unprotonated form fluoresces very little. The experiments involving CH_3-H_4-folate were done at pH 7.6, and the pK_a for CH_3-H_4folate is 4.88. Fluorescence data were digitally corrected by subtracting the very weak fluorescence spectrum of the CH_3-H_4folate solution from that of the solution containing enzyme plus CH_3-H_4folate. The bis-ANS concentration was determined using an extinction coefficient at 386 nm of 22 mM^{-1} cm^{-1} (Farris *et al.*, 1978). The relative fractional quenching was calculated by dividing the decrease in emission intensity at a particular concentration of quencher by the maximum decrease in intensity. The emission intensity was corrected for quantum yield (Parker & Rees, 1960).

Pre-Steady State Kinetics. Pre-steady-state kinetic experiments were performed at 25 °C with a DX.17MV sequential stopped-flow ASVD spectrofluorimeter from Applied Photophysics (England) under anaerobic conditions as described earlier (Zhao *et al.*, 1995). The C/Fe-SP was reduced by incubating 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, in a tonometer. 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 nonzero base line. For

$$A = Ce^{-kt} + b \qquad (2)$$

studying inhibition of the MeTr reaction by bis-ANS, a solution containing bis-ANS and MeTr was rapidly mixed with the C/Fe-SP and H_4folate. We observed the reaction with bis-ANS at 450 and 550 nm only, since bis-ANS has a strong absorption at 390 nm.

Stopped-Flow Fluorescence Studies. A solution containing MeTr and bis-ANS in 2 mM Tris-HCl, pH 7.6, 0.1 M NaCl was rapidly mixed with 50 mM MES or potassium succinate buffer at 25 °C at different pH values. The solution was excited at 405 nm using a 1 mm slit width. The emission intensity was observed as photomultiplier tube voltage using a 420 nm cutoff filter from Oriel Corp. The cuvette path length was 2 mm. All data were fit to a double-exponential equation (eq 3), where C_1 and C_2 are the amplitudes, k_1 and

$$V = C_1e^{-k_1t} + C_2e^{-k_2t} + b \qquad (3)$$

k_2 are the rate constants, V is the photomultiplier tube voltage, and t is the time. In the kinetic traces, one observes a decrease in voltage as the fluorescence increases because the stopped-flow instrument measures transmitted light.

RESULTS AND DISCUSSION

Binding of (6S)-CH_3-H_4folate to MeTr. When excited at 295 nm, MeTr exhibits a fluorescence emission spectrum

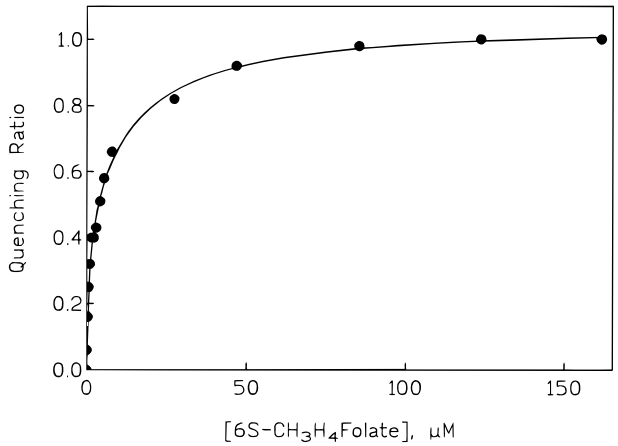

FIGURE 1: Binding of (*6S*)-CH_3-H_4folate to MeTr. (*6S*)-CH_3-H_4-folate was added anaerobically to a solution containing 0.5 μM MeTr in 50 mM Tris-HCl, pH 7.6. The tryptophan fluorescence was determined by exciting at 295 nm and measuring the emission intensity at 344 nm. The quenching ratio was plotted against the concentration of CH_3-H_4folate and fitted to the equation for a double rectangular hyperbola using the program Inplot 4: $[AX/(K_{d1} + X)] + [CX/(K_{d2} + X)]$, where Y is the quenching ratio (the observed fluorescence quenching at a particular CH_3-H_4folate concentration divided by the maximum quenching at a saturating CH_3-H_4folate concentration), X is the total concentration of CH_3-H_4folate, and A and C are the maximum quenching ratios at the two sites. The fit values were $K_{d1} = 0.64 \pm 0.2$ μM, $K_{d2} = 13 \pm 4$ μM, $A = 0.42 \pm 0.08$, and $C = 0.64 \pm 0.07$.

with a maximum between 344 and 351 nm. The absorption and emission profiles are characteristic of intrinsic fluorescence from tryptophan residues (Lakowicz, 1986). MeTr has two tryptophan residues per monomer (Roberts *et al.*, 1994) Upon incubation of (*6S*)-CH_3-H_4folate with MeTr, the intrinsic tryptophan fluorescence at 344 nm was quenched and the fluorescence of CH_3-H_4folate at 360 nm was enhanced. By following the fluorescence quenching at 344 nm at different concentrations of CH_3-H_4folate, a biphasic curve was observed. Fitting the data to a two-site binding model gave K_d values of 0.6 and 13 μM at pH 7.6 (Figure 1). We could not measure the K_d at low pH values because, under these conditions, the fluorescence of CH_3-H_4folate was much more intense than that of MeTr.

The fluorescence quenching by CH_3-H_4folate was attributed to binding of substrate to the active site because the dissociation constant for the CH_3-H_4folate-MeTr complex (0.6 μM) is similar to the K_m values determined by steady-state kinetic experiments ($\leq$10 μM) (Roberts, 1992). Thus, at least one of the two tryptophans in the protein is involved in binding CH_3-H_4folate. A plausible quenching mechanism is energy transfer between the pterin ring and this closely spaced tryptophan(s). Such an arrangement is reminiscent of interactions between pterins and aromatic residues in other proteins. In thymidylate synthase, two tryptophan residues are close to the methylene-H_4folate pterin ring (Montfort *et al.*, 1990). A tryptophan residue that is part of the H_2folate binding pocket in dihydrofolate reductase appears to play a role in transition-state binding; mutation of this residue resulted in weakened H_2folate binding and a decreased rate of hydride transfer (Warren *et al.*, 1991). In addition, the side chain of a phenylalanine residue in dihydrofolate reductase is perpendicular to the *p*-aminobenzoyl group of folate (Stammers *et al.*, 1987; Oefner *et al.*, 1988). Mutation of this residue results in a 100−1000-fold weaker methotrexate binding affinity (Stammers *et al.*, 1987; Thillet *et al.*, 1988).

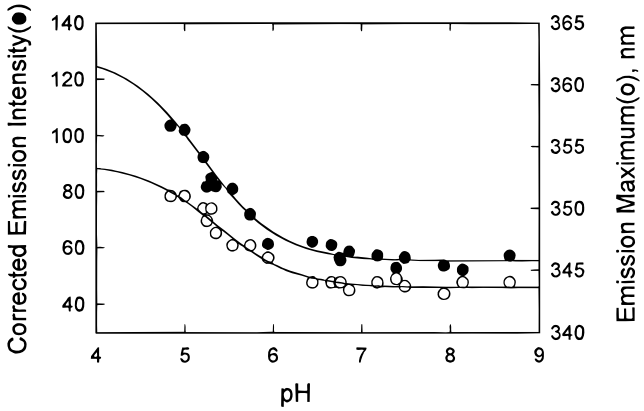

FIGURE 2: pH-dependent conformational change in MeTr followed by intrinsic tryptophan fluorescence. A solution containing 0.5 μM MeTr in 50 mM MES, potassium succinate, or HEPES buffer was excited at 295 nm. The ionic strength of the buffer was maintained at 0.1 M by adding appropriate amounts of NaCl. The emission intensity and emission maximum were monitored, plotted against pH, and fitted to the equation $Y = A/[1 + 10^{(pH-pK_a)}] + B$, where B is an offset value to account for a nonzero base line and A is the maximum difference between the values at high and low pH. The emission intensity was corrected for quantum yield (Parker & Rees, 1960). The best-fit values for the emission intensity were $A = 74 \pm 9$, $pK_a = 5.2 \pm 0.1$, and $B = 55 \pm 1$. For the emission maximum the best fit values were $A = 10 \pm 1$, $pK_a = 5.4 \pm 0.1$, and $B = 344 \pm 0.2$.

pH-Dependent Conformational Change. Steady-state and pre-steady-state kinetic studies demonstrate that the rate of the MeTr reaction increases as the pH is lowered (Zhao *et al.*, 1995). As the pH was lowered into the range of the kinetic pK_a (between pH 5.0 and 5.3), the tryptophan fluorescence emission intensity was enhanced and the emission maximum underwent a red shift (Figure 2). The pH profiles for the changes in the emission maximum and in the emission intensity fitted well to single-titration curves with pK_a values of 5.4 and 5.2, respectively. These changes in intrinsic tryptophan fluorescence indicate that MeTr undergoes a conformational change in which a hydrophobic region containing a buried tryptophan residue(s) becomes more solvent accessible at pH values near the kinetic pK_a.[2] Because of the fluorescence quenching by CH_3-H_4folate, the tryptophan-containing region appears to be involved in substrate binding.

Bis-ANS was used to further study the MeTr conformational change since its fluorescence intensity is enhanced when it binds to the hydrophobic residues of proteins (Farris *et al.*, 1978). Quenching of the intrinsic tryptophan fluorescence of MeTr was accompanied by enhancement of the bis-ANS fluorescence upon binding MeTr (Figures 3A and 4, inset). The bis-ANS emission maximum underwent a blue shift as the pH was lowered, moving from 494 nm at pH 5.4 to 508 nm at pH 7.9. The pH profile for the changes in fluorescence intensity of the bis-ANS−MeTr complex fit a single titration with a pK_a value of 5.0 (Figure 3B). This value is similar to the pK_a value measured for tryptophan fluorescence enhancement indicating that the

[2] We could not measure fluorescence data below pH 4.8 because MeTr precipitates at its isoelectric point which is pH 4.8 (Roberts, 1992). This is near its kinetic pK_a, which is between pH 5.0 and 5.3 (Zhao *et al.*, 1995).

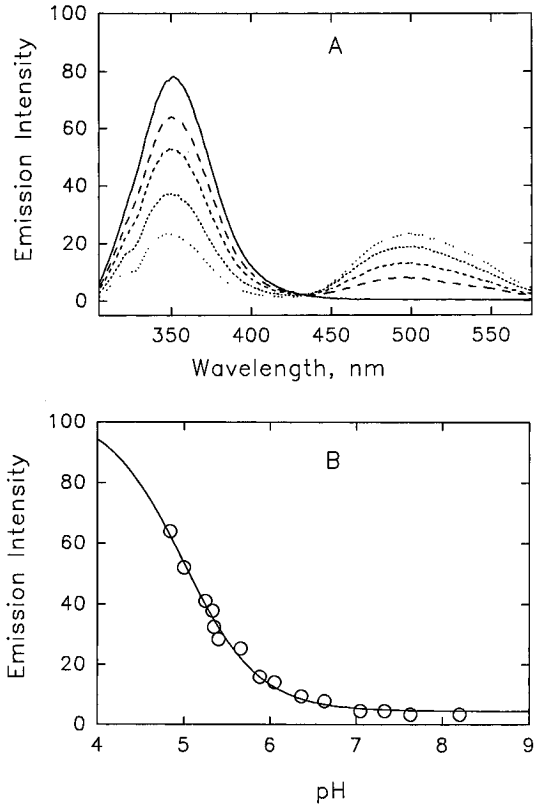

FIGURE 3: Binding of bis-ANS to MeTr. (A) A solution containing 0.5 μM MeTr in 50 mM MES, pH 5.36, and varying amounts of bis-ANS (0, 2.5 , 5.04, 10.1, and 17 μM) was excited at 295 nm. The MeTr fluorescence at ~350 nm was quenched as the bis-ANS fluorescence at ~494 nm was enhanced. The free bis-ANS spectra were subtracted from the spectra. (B) A 1 μM MeTr solution was incubated with 50 μM bis-ANS in 50 mM potassium succinate, MES, or Tris-HCl buffer at different pH values. After excitation at 405 nM, the emission intensity was monitored. The ionic strength of the buffer was maintained at 0.1 M by adding appropriate amounts of NaCl. The emission intensity was plotted against the pH and fitted to the pH titration equation given in the Figure 2 legend. The best-fit values for the emission intensity were $A = 99 \pm 9$, p$K_a = 5.0 \pm 0.1$, and $B = 4.4 \pm 0.9$.

same process is being observed by both methods. The blue shift in the bis-ANS emission maximum indicates that a hydrophobic region becomes more solvent accessible at low pH.

Enhancement of the bis-ANS fluorescence was used to quantitate the number of binding sites and the dissociation constant for bis-ANS. First, a calibration factor (Klotz & Hunston, 1971) was determined to relate the maximum fluorescence increase per μM of bis-ANS bound to MeTr. By varying the MeTr concentration from 0 to 5 μM at a constant concentration of bis-ANS (30 μM), the calibration factor was determined to be 15.28 at pH 5.4 and 1.58 at pH 7.9. Using this factor, the apparent K_d for binding of bis-ANS to MeTr was found to be 1.74 μM with 3.1 binding sites at pH 5.36 (Figure 4) and 410 $\pm$ 200 μM with 3.63 $\pm$ 0.05 binding sites at pH 7.93. Thus, lowering the pH by 2.5 units increases the affinity of MeTr for bis-ANS by over 100-fold. The effect of pH on bis-ANS binding to this hydrophobic region should correlate with that on CH_3-H_4-folate binding. We were unable to determine the pH dependence of the dissociation constant for the CH_3-H_4-folate−MeTr complex directly because the fluorescence from CH_3-H_4folate obscures any quenching of the tryptophan fluorescence. Since bis-ANS binds over 200-fold weaker

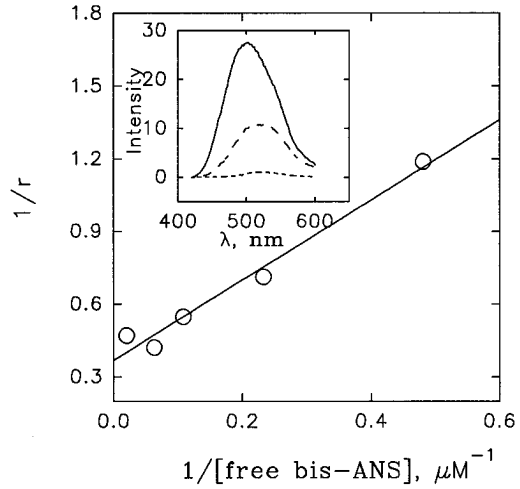

FIGURE 4: Determination of the dissociation constant for bis-ANS and MeTr. Different amounts of bis-ANS were added to a solution containing 0.5 μM MeTr in 50 mM MES pH 5.36. The excitation wavelength was 405 nm, and the emission intensity at 494 nm was measured. A calibration factor of 15.28 arbitrary units of increased fluorescence per μM bis-ANS bound to a given concentration of MeTr was determined. The data were then fit to the equation $1/r = 1/n + (1/nK_{app})(1/[\text{bis-ANS}]_f)$, where r is the number of moles of bis-ANS bound per mole of MeTr, n is the number of binding sites, K_{app} is the apparent dissociation constant, and $[\text{bis-ANS}]_f$ is the concentration of free bis-ANS. The values of n and K_{app} were respectively 3.1 $\pm$ 0.2 and 1.7 $\pm$ 0.2 μM. Inset: Emission spectra after excitation at 405 nm.

at pH 7.9 than at pH 5.5, the binding constant for CH_3-H_4-folate is likely to be similarly affected by pH.

Inhibition of MeTr by Bis-ANS. The transmethylation reaction can be followed at 390 nm ($\epsilon = 30$ mM^{-1} cm^{-1}) or 550 nm ($\epsilon = 7.5$ mM^{-1} cm^{-1}) where cob(I)amide absorbs or at 450 nm ($\epsilon = 12.5$ mM^{-1} cm^{-1}) where methylcob(III)amide absorbs. The rate constant for methylation of the C/Fe-SP was shown earlier by stopped-flow kinetics to be 22 s^{-1} (Zhao *et al.*, 1995). We followed the rate of methylation of the C/Fe-SP at pH 5.8 at varied concentrations of bis-ANS (Figure 5). The data were fitted to an inhibition equation to yield a K_i value of 26 μM. This value agrees well with the pH-dependent K_d determined by fluorescence enhancement studies (above).

Stopped-Flow Fluorescence Studies. The above studies indicate that MeTr undergoes a pH-dependent conformational change. It was important to evaluate its kinetic relevance. The rate at which the conformational change occurs was followed by measuring the rate of fluorescence enhancement at saturating concentrations of bis-ANS (Figure 6A) where the conformational change, not binding of bis-ANS, is rate-limiting. Thus, the measured rate constant should equal the rate constant for the conformational change. The data fit a double-exponential equation with observed rate constants of 40 and 5.7 s^{-1}. The rate constant for the first phase was slightly higher than the k_{cat} for the MeTr reaction, 18 s^{-1}, and the pre-steady-state derived rate constants for the transmethylation reactions in the forward (25 s^{-1}) and reverse (40 s^{-1}) directions (Zhao *et al.*, 1995). These results indicate that the conformational change is kinetically competent. The second phase was too slow to be kinetically relevant and could reflect nonspecific binding of bis-ANS.

Surprisingly, when a MeTr-bis-ANS solution was mixed with buffers at different pH values, the rate constants were

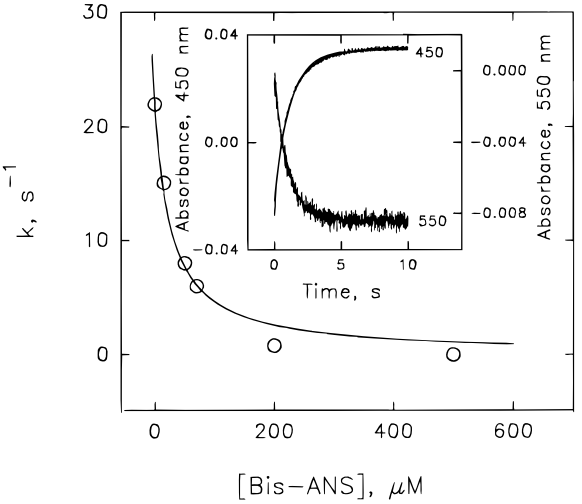

FIGURE 5: Inhibition by bis-ANS of the methyl transfer reaction. Solutions containing 20 μM MeTr, 10 mM Tris-maleate, pH 5.8, 100 mM NaCl, and different concentrations of bis-ANS were reacted with a solution containing 15 μM Co^{1+}−C/Fe-SP, 100 μM (*6S*)-CH_3-H_4folate in 1 mM Tris-HCl, pH 7.6. The absorbance decrease at 550 nm and increase at 450 nm were fitted to single-exponential equations. The rate constant for the reactions followed 450 nm were plotted against the bis-ANS concentration, and the data were fitted to the equation $k = k_{max}K_i/(K_i + [\text{bis-ANS}])$, to yield k_{max}, the rate at 0 μM bis-ANS, of 22.3 ± 1.1 s^{-1} and K_i, the inhibition constant, of 26.2 ± 4.2 μM. Inset: Kinetic traces at 450 and 550 nm. Reaction conditions were as just outlined except the the concentration of bis-ANS was 400 μM. The absorbance decrease at 550 nm and increase at 450 nm were fitted to single-exponential equations. The calculated rate constants were k_{450} = 0.8 ± 0.05 s^{-1} and k_{550} = 0.8 ± 0.01 s^{-1}.

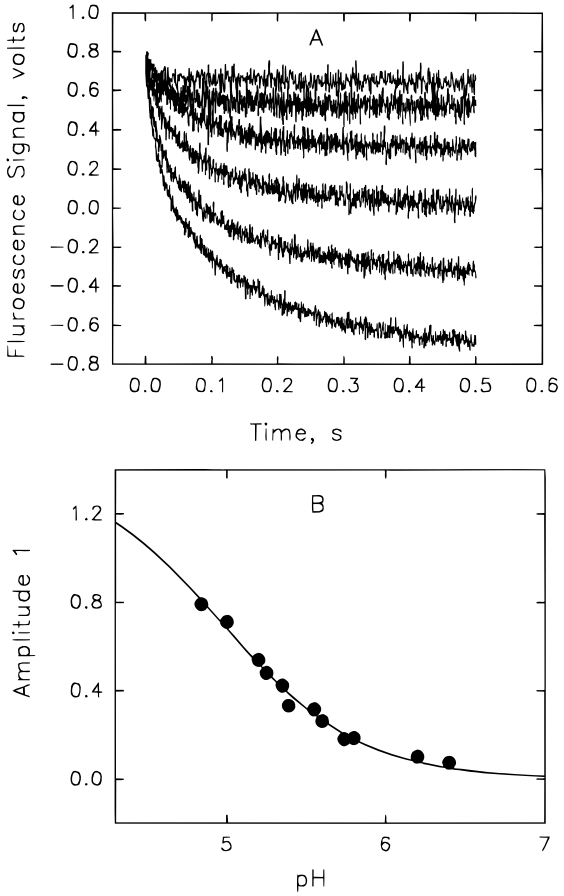

FIGURE 6: Kinetic competence of the conformational change. A solution containing 1 μM MeTr, 20 μM bis-ANS, 2 mM Tris-HCl, pH 7.6, 0.1 M NaCl was rapidly mixed with 50 mM MES or potassium succinate buffer with the stopped-flow instrument at 25 °C. The stopped flow was in the fluorescence mode. The solution was excited at 405 nm, and the emission above 420 nm was collected. (A) Fluorescence traces at different pH. The rate constants and amplitudes were determined by fitting to double-exponential equations. The rate constants were independent of pH with a k_1 of 40 ± 5 s^{-1} and k_2 of 5.7 ± 0.4 s^{-1}. (B) The amplitude of the first phase was plotted against pH to give a pK_a of 5.0 ± 0.1 and a maximum amplitude of 1.4 ± 0.1.

independent of pH. The amplitudes for both phases increased as the pH was decreased (Figure 6A). The pH profiles for the changes in amplitude of the first phases fit a single-titration curve with a pK_a of 5.0 (Figure 6B), which closely matches the pK_a values for the static fluorescence measurements and for the kinetics of the forward and reverse reactions. In addition, the rate constant is similar to the k_{cat} for both overall reactions. These results indicate that only the protonated form of MeTr is active and that this form varies according to a pK_a of ∼5.0.

CONCLUSIONS

On the basis of these results and the earlier studies that rule out protonation of CH_3-H_4folate as the rate-limiting proton transfer (Zhao *et al.*, 1995), we propose that a pH-dependent conformational change in MeTr is a kinetically competent intermediate step in the MeTr mechanism. Furthermore, it appears to be the primary rate-limiting step in both the methylation of the C/Fe-SP by CH_3-H_4folate (forward reaction) and the demethylation of the methylated C/Fe-SP by H_4folate (reverse reaction). Rate-limiting conformational changes have been observed in other systems. A rate-limiting conformational change in the α subunit of the heterotrimeric G protein appears to result in its deactivation (Neubig *et al.*, 1994). The rate-limiting step in the fumarase reaction appears to be interconversion of the enzyme between its two conformers (substrate-specific and product-specific forms) (Rose *et al.*, 1993).

Our results do not reject or support the hypothesis that protonation of CH_3-H_4folate is important in the reaction. The protonation state of bound CH_3-H_4folate could be addressed by other studies, such as NMR. The results reported here and earlier (Zhao *et al.*, 1995) demonstrate that this proton transfer, if it occurs, must be faster than the one leading to the MeTr conformational change.

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