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Participation of Cob(I)alamin in the Reaction Catalyzed by Methionine Synthase from *Escherichia coli*: A Steady-State and Rapid Reaction Kinetic Analysis†

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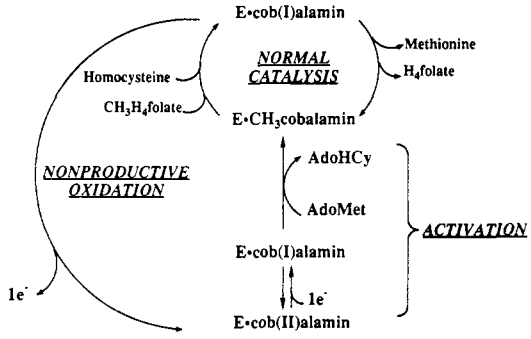
ABSTRACT: The kinetic mechanism of the reaction catalyzed by cobalamin-dependent methionine synthase from *Escherichia coli* K12 has been investigated by both steady-state and pre-steady-state kinetic analyses. The reaction catalyzed by methionine synthase involves the transfer of a methyl group from methyltetrahydrofolate to homocysteine to generate tetrahydrofolate and methionine. The postulated reaction mechanism invokes an initial transfer of the methyl group to the enzyme to generate enzyme-bound methylcobalamin and tetrahydrofolate. Enzyme-bound methylcobalamin then donates its methyl group to homocysteine to generate methionine and cob(I)alamin. The key questions that were addressed in this study were the following: (1) Does the reaction involve a sequential or ping-pong mechanism? (2) Is enzyme-bound cob(I)alamin a kinetically competent intermediate? (3) If the reaction does involve a sequential mechanism, what is the nature of the "free" enzyme to which the substrates bind; i.e., is the prosthetic group in the cob(I)alamin or methylcobalamin state? Both the steady-state and rapid reaction studies were conducted at 25 °C under anaerobic conditions. Initial velocity analysis under steady-state conditions revealed a family of parallel lines suggesting either a ping-pong mechanism or an ordered sequential mechanism. Steady-state product inhibition studies provided evidence for an ordered sequential mechanism in which the first substrate to bind is methyltetrahydrofolate and the last product to be released is tetrahydrofolate. Pre-steady-state kinetic studies were then conducted to determine the rate constants for the various reactions. Enzyme-bound cob(I)alamin was shown to react very rapidly with methyltetrahydrofolate (with an observed rate constant of 250 s^{-1} versus a turnover number under maximal velocity conditions of 19 s^{-1}). Enzyme-bound cob(I)alamin was also shown to form rapidly (140 s^{-1}) when homocysteine was mixed with methylated enzyme, thus establishing its kinetic relevance as an intermediate. A minimal kinetic mechanism that accommodates the steady-state data and the measured rate constants was employed to simulate the kinetic behavior of the system. The simulations made very different predictions depending on whether the prosthetic group was in the cob(I)alamin or methylcobalamin state in "free" enzyme and yielded an excellent fit to the real data only when enzyme containing bound methylcobalamin was employed as the starting enzyme. From these studies it was concluded that methylcobalamin-containing methionine synthase is the "free" form of the enzyme, to which the substrates bind in an ordered sequential fashion, and that enzyme-bound cob(I)alamin is a kinetically competent intermediate.

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH_3-H_4folate)[1] to homocysteine, generating H_4folate and methionine as shown in eq 1.

$$CH_3\text{-}H_4\text{folate} + \text{homocysteine} \rightarrow H_4\text{folate} + \text{methionine} \quad (1)$$

The enzyme has been the subject of a recent review (Banerjee & Matthews, 1990). The *metH* gene from *Escherichia coli*, which encodes cobalamin-dependent methionine synthase, has been cloned and sequenced and its gene product overexpressed (Banerjee et al., 1989; Old et al., 1990). The enzyme contains a noncovalently bound cobalamin prosthetic group that functions as an intermediary in the methyl-transfer reaction as shown in Scheme I. During catalysis the enzyme shuttles between the E·methylcobalamin and E·cob(I)alamin states, being alternately demethylated by homocysteine and remethylated by CH_3-H_4folate. The observed net retention of configuration for the enzyme-catalyzed transfer of a chiral methyl group from CH_3-H_4folate to methionine (Zydowsky et al., 1986) is consistent with a double displacement of the methyl group with transient residence on cobalamin. Under the steady-state assay conditions, the moderately labile E·cob(I)alamin intermediate is occasionally oxidized to E·cob-

Scheme I: Postulated Reaction Mechanism of Methionine Synthase Showing the Interconversion of the Various Oxidation States of E·Cobalamin



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[1] Abbreviations: CH_3-H_4folate, 5-methyl-5,6,7,8-tetrahydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; AdoMet, *S*-adenosylmethionine; AdoHCy, *S*-adenosylhomocysteine; E·cobalamin, enzyme-bound cobalamin.

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(II)alamin, which needs to be activated by a reductive methylation reaction to return the enzyme to the catalytic cycle as shown in Scheme I.

While the formation of both E·methylcobalamin and E·cob(I)alamin intermediates on the enzyme has been demonstrated (Taylor & Weissbach, 1969a; Taylor & Hanna, 1970; Fujii & Huennekens, 1979), the kinetic competence of these intermediates has not been established. Furthermore, the kinetic mechanism of the *E. coli* enzyme has not been previously elucidated, although very limited kinetic studies of the enzyme isolated from the pig kidney were performed by Burke et al. (1971). In this paper we describe steady-state and pre-steady-state kinetic analyses of the transmethylation reaction catalyzed by methionine synthase. The present study provides evidence for the participation of a ternary complex in catalysis and for the kinetic competence of E·cob(I)alamin as an intermediate along the reaction coordinate. In addition, our studies demonstrate that the "free" form of the enzyme, to which substrates bind, is E·methylcobalamin.

Experimental Procedures

Materials. Methionine synthase was purified from *E. coli* K-12 as described previously (Banerjee et al., 1989). AdoMet (iodide salt), aquocobalamin, and L-homocysteine thiolactone were purchased from Sigma. Dithiothreitol was from Bio-Rad. (6-*ambo*)-[methyl-^{14}C]CH_3-H_4folate (barium salt, 59 mCi/mmol) was purchased from Amersham. (6-*ambo*)-CH_3-H_4-folate (calcium salt) was obtained from Sapec Chemical Co. (Switzerland). (6-*ambo*)-H_4folate was purchased from Schirck (Switzerland), while the 6*S* isomers of both CH_3-H_4folate and H_4folate were prepared as described previously (Matthews et al., 1982).

Steady-State Kinetic Experiments. The steady-state kinetic experiments were performed using one of two methods. A continuous assay in which H_4folate formation was monitored was performed in a temperature-controlled stopped-flow spectrophotometer interfaced with a Nova 2 (Data General) microcomputer system described by Beaty and Ballou (1981). The stopped-flow observation path was 2 cm. The experiments were performed by mixing an anaerobic solution of enzyme with an anaerobic solution containing substrates and monitoring the increase in absorbance at 321 nm associated with the conversion of CH_3-H_4folate to H_4folate. Methylated methionine synthase (0.1 μM, before mixing) in 20 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol and AdoMet (19 μM), was made anaerobic in a tonometer in which dithiothreitol (25 mM) and aquocobalamin (10 μM) were placed in a side arm. Dithiothreitol was included in the enzyme solution, as it was found to enhance the stability of the methylcobalamin form of the enzyme under anaerobic conditions. The enzyme solution was made anaerobic by taking it through several cycles of alternately evacuating and flushing with purified argon followed by equilibration with argon for 5 min at 4 °C. This procedure was repeated eight times over approximately 50 min. Dithiothreitol and aquocobalamin were then tipped in and the enzyme was allowed to warm to room temperature to avoid outgassing during the course of the experiment. The substrate mixture was made up in a syringe in buffer containing 20 mM potassium phosphate buffer, pH 7.2/20% glycerol and made anaerobic by bubbling with purified argon for 15 min. The solutions were then transferred to the stopped-flow apparatus and equilibrated for ca. 3 min at 25 °C. The concentration of enzyme was chosen so that the reaction took place over approximately 10–60 s. The steady-state kinetic results are expressed as turnover numbers, i.e., the number of moles of H_4folate formed per second per mole of enzyme-bound cobalamin. Since the high absorbance of both CH_3-H_4folate and H_4folate at 321 nm limited the concentration range of the folate substrate and product that could be employed, the product inhibition studies were repeated by using the standard fixed time radiochemical assay at 37 °C described by Frasca et al. (1988).

Rapid-Reaction Kinetic Experiments. Whereas the standard in vitro assay requires an activation system (dithiothreitol, aquocobalamin, and AdoMet) and is conducted under semianaerobic conditions, the activated enzyme that has the prosthetic group in the methylcobalamin state can be assayed directly by mixing with substrates under rigorously anaerobic conditions in the stopped-flow spectrophotometer. Under these conditions, the assay mixture is simpler in composition and lacks both AdoMet and aquocobalamin, which interfere with the spectral monitoring of the enzyme. This system therefore permits a continuous assay where multiple turnovers can be monitored by observing the enzyme-bound cobalamin with UV–visible absorption spectroscopy. All stopped-flow experiments were performed at 25 °C under anaerobic conditions by using procedures described above. The concentration of methylated enzyme used in all experiments was 10 μM (before mixing) and it was made up in 20 mM potassium phosphate buffer, pH 7.2. Dithiothreitol (25 mM) was placed in a side arm and tipped in after the solution was made anaerobic. The enzyme was protected from light as the Co–methyl bond in enzyme-bound methionine synthase is somewhat light sensitive. For experiments in which the reactivity of E·cob(I)alamin was studied, E·cob(I)alamin was generated by introducing anaerobic homocysteine or H_4folate via an air-tight Hamilton syringe with a long needle into the tonometer containing anaerobic methylated methionine synthase. The pink color associated with the methylated enzyme rapidly disappeared upon addition of homocysteine or H_4folate. The subsequent experiments had to be performed rapidly due to the lability of E·cob(I)alamin, which oxidizes to E·cob(II)alamin under these conditions with a half-time of approximately 30 min.

Methylation of Methionine Synthase. Methionine synthase was methylated by an electrochemical method as described by Banerjee et al. (1990). The purified enzyme contains the prosthetic group predominantly in the methylcobalamin and cob(II)alamin states (Banerjee et al., 1989) and was converted completely to the methylcobalamin form by mixing enzyme (ca. 100 nmol in 1 mL of 20 mM potassium phosphate buffer, pH 7.2/100 mM KCl) with AdoMet (1 mM) and methylviologen (10 mM). The solution was made anaerobic by cycles of vacuum and nitrogen gas, and the potential was poised at −450 mV (versus the standard hydrogen electrode) for an hour. The solution was kept protected from light during this and subsequent handling procedures due to the light sensitivity of methylated methionine synthase. The enzyme was concentrated in a Centricon 30 microconcentrator (Amicon) and washed with 10 volumes of 20 mM potassium phosphate buffer, pH 7.2, prior to the final concentration. The activity of the enzyme was monitored before and after the methylation reaction, and no difference in the specific activity was observed.

Data Analysis. Rate constants were obtained from exponential fits to the data by using a nonlinear least-square fitting routine based on the Marquardt algorithm (Bevington, 1969) developed by Dr. C. Batie at the Biological Chemistry Department of The University of Michigan. The kinetic mechanisms depicted in Scheme III were simulated on the Nova 2 minicomputer using fourth-order Runge-Kutta techniques, by calculating concentrations of reactants, intermediates, and products as a function of time. These concentrations were

Table I: Steady-State Kinetic Parameters for Methionine Synthase in 20 mM Potassium Phosphate Buffer, pH 7.2, Containing 20% Glycerol[a]

kinetic parameter	value
k_{cat} (s^{-1})	18.8 ± 3[b]
K_m [(6S)-CH_3-H_4folate] (μM)	27.8 ± 6.3[b]
K_m (homocysteine) (μM)	0.8 ± 5.9[b]
K_i [(6S)-CH_3-H_4folate] (μM)	60 ± 507[b]
K_{on} [(6S)-CH_3-H_4folate] (M^{-1} s^{-1})	(0.67 ± 0.05) × 10^6[c]

[a] Steady-state data were obtained at 25 °C in the stopped-flow apparatus as described under Experimental Procedures. [b] These values were derived by application of the SEQUENO program of Cleland (1979). [c] Calculated by using the formula for propogation of errors of Bevington (1969).

computed by numeric solutions of the set of differential equations describing the mechanisms depicted in Scheme III with the input of values for initial reactant concentrations, rate constants, and extinction coefficients (Beaty & Ballou, 1981).

Results

Initial Velocity Steady-State Kinetic Data. The general initial rate equation for an enzyme-catalyzed reaction involving two substrates, as described by Cleland (1963), is given by eq 2. Initial estimates of the kinetic constants K_{mA}, K_{mB}, K_{dA},

$$\frac{V_{max}}{v} = 1 + \frac{K_{mA}}{[A]} + \frac{K_{mB}}{[B]} + \frac{K_{dA}K_{mB}}{[A][B]} \quad (2)$$

and V_{max} can be obtained by primary intercept and primary slope replots of the data obtained from Lineweaver–Burk plots. More accurate values and standard errors were obtained using the SEQUENO program of Cleland (1979). A and B represent the first (CH_3-H_4folate) and second substrate (homocysteine), respectively, to bind to the enzyme, as discussed under the product inhibition studies. Data from an experiment in which the concentration of homocysteine was varied at fixed concentrations of CH_3-H_4folate are shown in Figure 1. A set of parallel lines with a very shallow slope was seen, indicating a low K_m for homocysteine. The values of the kinetic constants for the reaction mechanism can be estimated from secondary plots of the initial rate data (Dalziel, 1957). Plots of this nature are shown in the insets of Figure 1, and the kinetic constants determined from such experiments are summarized in Table I.

A set of parallel lines in the analysis of initial velocity steady-state kinetic data is generally indicative of a nonsequential or ping-pong mechanism, in which the second substrate binds only after the first product has been released. However, under certain restricting conditions (when $K_{dA} \ll K_{mA}$ and/or $K_{mB} \ll$ [B]), the slopes of the plots become insensitive to changes in the concentration of the second substrate and the plots are essentially parallel for a sequential mechanism (Dalziel, 1962). Our data fit into this category and thus give unreliable results on the slope replot (data not shown).

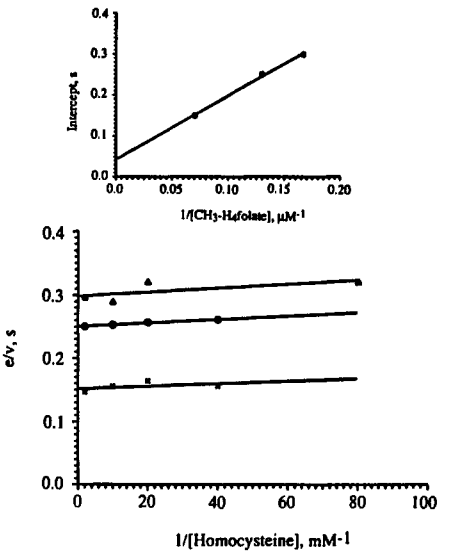


FIGURE 1: Steady-state kinetic analysis of methionine synthase catalysis with varying homocysteine concentrations. The conditions are described under Experimental Procedures. The concentration of the natural 6S isomer of CH_3-H_4folate is 6.25, 7.5, and 15 μM for the upper, middle, and lower traces, respectively. Inset: Primary intercept replot versus the reciprocal CH_3-H_4folate concentration.

Product inhibition studies are then employed to distinguish between the two mechanisms.

Product Inhibition Data. The results from the product inhibition analysis are given in Table II. The high extinction coefficient of both CH_3-H_4folate and H_4folate limited the substrate (40–100 μM) and product (0–200 μM) concentration ranges that could be employed in the continuous spectrophotometric assay. Hence, these studies were repeated with a fixed-time radiochemical assay (data not shown) that is routinely employed for assaying the enzyme, in which the transfer of a ^{14}C-labeled methyl group from CH_3-H_4folate to methionine is monitored. The concentration ranges for CH_3-H_4folate and H_4folate used in these experiments were 14.8–148 and 0–375 μM, respectively. The same product inhibition patterns were obtained, in addition to identical values for K_m for CH_3-H_4folate (ca. 25 μM in each case). The K_m for homocysteine was extremely low and could not be measured accurately since the assays were not sufficiently sensitive to monitor product formation at concentrations below the K_m for homocysteine. The K_i for H_4folate obtained from the fixed-time assay was ca. 1 mM. The K_i for methionine obtained from both assays was 1.1 mM.

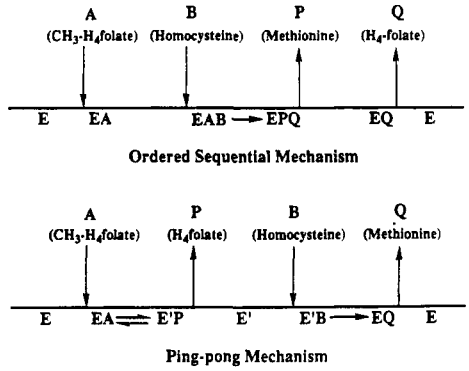
The predicted patterns for standard product inhibition analysis in a bi-bi mechanism are predicated on the ability of the product inhibitors to reverse the catalytic sequence. The reaction catalyzed by methionine synthase is, however, irreversible as E·cob(I)alamin is unable to accept a methyl group from methionine. Hence, the predictions for the product in-

Table II: Product and Dead-End Inhibition Patterns in Phosphate Buffer, pH 7.2, Containing 20% Glycerol

inhibitor	varied substrate	fixed substrate	predicted pattern for ordered sequential[c] product	predicted pattern for ordered sequential[c] dead-end[a]	predicted pattern for ping-pong[c] product	predicted pattern for ping-pong[c] dead-end[b]	obsd pattern
L-Met	CH_3FH_4	HCy (satd)	U	U (EA/EQ)	C	C (E)	U
L-Met	HCy	CH_3FH_4 (satd)	M	C (EA), U (EQ) M (EA/EQ)	M	U (E) M (E,E′)	M
FH_4	CH_3FH_4	HCy (satd)	C	C (E)	C (E′)	C (E′)	C
FH_4	HCy	CH_3FH_4 (unsatd)	M	M (E)	M (E′)	M (E′)	M

[a] The forms of the enzyme to which the inhibitors bind are indicated in parentheses and refer to the species described in Scheme II. [b] E′ refers to E·methylcobalamin; E refers to E·cob(I)alamin. U, M, and C refer to uncompetitive, mixed, and competitive inhibition, respectively. [c] See Scheme II.

Scheme II: Description of Ordered Sequential and Ping-Pong Kinetic Mechanisms



hibition patterns based on the assumption that the products of methionine synthase inhibit by binding as dead-end inhibitors, and the assumed enzyme form to which the product binds, have been included in Table II for both ping-pong and ordered-sequential mechanisms (as described in Scheme II). The predictions for the dead-end complexes are based on the simplistic assumption that a product may bind to one form of the enzyme or the other, but not to both forms. The observed uncompetitive inhibition by methionine when CH_3-H_4folate is the varied substrate at saturating homocysteine concentrations (Table II) clearly distinguishes between an irreversible or dead-end ping-pong mechanism and an ordered sequential mechanism. When methionine is the inhibitor and homocysteine is the varied substrate, mixed inhibition is observed and could fit either mechanism if it is assumed that methionine can bind to E and to E′ (in the ping-pong mechanism) or to EA and EQ (in the ternary mechanism). The patterns obtained when H_4folate is used as the inhibitor do not discriminate between the two mechanisms.

Thus, our product inhibition data distinguish between an ordered ternary mechanism and a ping-pong mechanism. A kinetic mechanism that agrees with these data must include a ternary complex of the enzyme, CH_3-H_4folate, and homocysteine. In addition, CH_3-H_4folate must be the first substrate that binds to the free enzyme and H_4folate must be the second product to dissociate in this ordered bi-bi system. The mechanism that is consistent with this analysis, written in the shorthand notation of Cleland (1963), is given in Scheme II.

Pre-Steady-State Kinetic Analysis of Catalysis. Kinetic events in the pre-steady-state phase of catalysis were studied by directly monitoring the spectral changes associated with the enzyme-bound methylcobalamin upon mixing substrates and methylated enzyme. The results of mixing homocysteine (500 μM) and (6*S*)-CH_3-H_4folate (200 μM) with methylated methionine synthase (5 μM) are shown in Figure 2. A very rapid initial increase in absorbance at 390 nm occurs (250 s^{-1}), corresponding to a rapid formation of E·cob(I)alamin. Then the absorbance decreases slightly over the next 20 ms as steady state is approached. Steady-state continues for 2–3 s, and at the end of the last turnover when CH_3-H_4folate is consumed, the enzyme accumulates as E·cob(I)alamin as shown by the large increase in absorbance at 390 nm over 15 s. Observations at 520 nm, where E·methylcobalamin absorbs more strongly, show similar patterns of the opposite sign.

The spectrum of the enzyme at the completion of the reaction was shown to be that of E·cob(I)alamin by UV–visible electronic absorbance spectroscopy. The complete conversion of 5 μM E·methylcobalamin to E·cob(I)alamin gives rise to a change in absorbance at 390 nm of 0.158 (measured in a cell with a 2-cm path length). The change in absorbance at

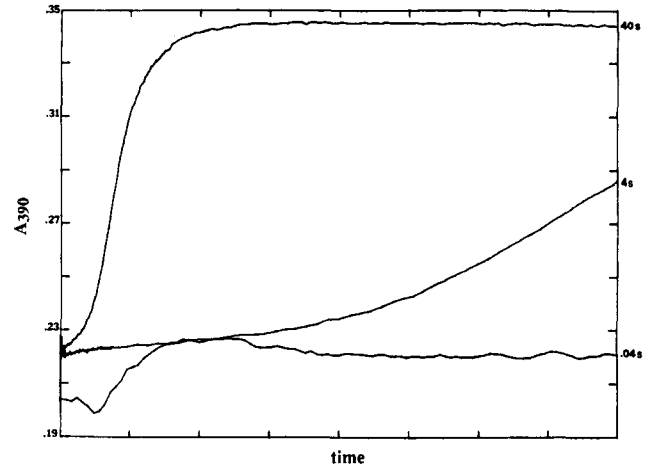


FIGURE 2: Changes in absorbance after mixing methylated methionine synthase (5 μM) with homocysteine (500 μM) and CH_3-H_4folate (200 μM). A pre-steady-state burst of cob(I)alamin formation is seen (by 10 ms), followed by a correction of the overshoot in the steady state. When CH_3-H_4folate is exhausted, the enzyme accumulates in the cob(I)alamin state. The reported optical density has not been corrected for the end absorbance due to CH_3-H_4folate and H_4folate.

390 nm as the catalytic reaction progressed from steady state (0.04 s) to the end of turnover (40 s) was 0.125, giving an estimate of 21% of the enzyme present as E·cob(I)alamin in steady-state turnover.

Pre-Steady-State Analysis of Turnover in the Presence of AdoMet. AdoMet is an essential component of the standard in vitro assay mixture for methionine synthase, where it donates a methyl group in the reductive activation of the enzyme. AdoMet has been shown to react with E·cob(I)alamin generated by photochemical reduction of E·cob(II)alamin (Banerjee et al., 1990). In order to determine whether AdoMet can intercept E·cob(I)alamin formed during turnover, the experiment described above was repeated in the presence of AdoMet (500 μM after mixing, added to the substrate syringe). AdoMet was found to affect neither the reaction rate nor the spectral composition of the enzyme during turnover (data not shown). From these results it may be concluded that cob(I)alamin is sequestered on the enzyme during turnover and is therefore inaccessible to AdoMet. These results are also consistent with the formation of a ternary complex during catalysis.

Spectrum of Methionine Synthase during Turnover. To verify that the data obtained by single-wavelength monitoring truly reflected the amount of E·cob(I)alamin during steady-state turnover, the spectral morphology of the enzyme during turnover was established directly by rapidly scanning between 320 and 720 nm. Methylated methionine synthase was rapidly mixed with the substrate mixture as described above. The spectrophotometer employed in this experiment scans from low to high wavelength at a rate of 60 nm/s; thus successive spectra staggered by 40 nm were recorded after each shot to be sure that the data were being recorded during the steady state. The end absorption contributed by CH_3-H_4folate was corrected by subtraction. A composite spectrum of the enzyme during turnover was then compiled from the overlapping component spectra and is shown in Figure 3. Approximately 70% of the enzyme is in the E·methylcobalamin state during turnover, with the remainder (30%) being in the E·cob(I)alamin state. These estimates are obtained by examining the absorbance changes at 520 nm where base-on E·methylcobalamin absorbs maximally. At 520 nm the end absorption due to CH_3-H_4folate and H_4folate is negligible, whereas at 390 nm spectral contribution from the folate substrate and product is substantial.

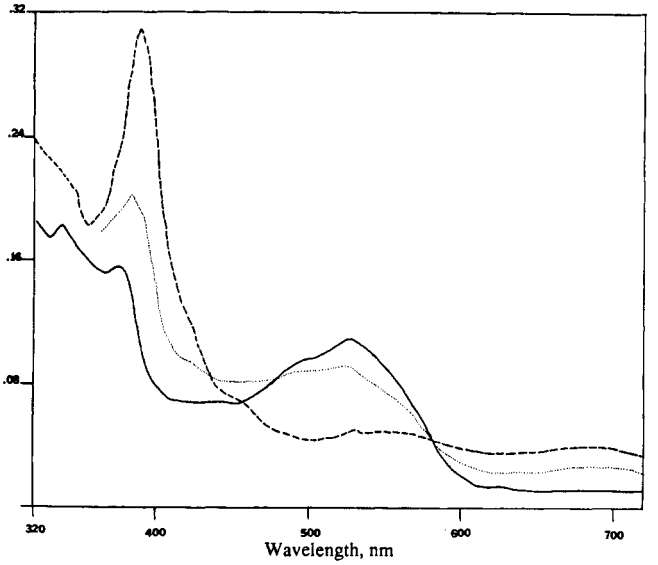


FIGURE 3: Spectra of methionine synthase before, during, and after turnover. The concentrations of reagents were the same as those described in Figure 2, and homocysteine was in excess. (—) Initial spectrum of enzyme in the methylcobalamin state; (···) spectrum of the enzyme immediately after mixing with substrates and obtained during turnover (this spectrum was compiled from several composite spectra as described under Results); (---) final spectrum of enzyme in the cob(I)alamin state when CH_3-H_4folate is exhausted. The reported optical density has been corrected for the end absorbance due to CH_3-H_4folate and H_4folate.

Due to the large correction that is required to subtract out the spectrum of the folate derivatives, the absolute absorbance at 390 nm is less accurate than that at 520 nm. A more careful examination of the spectrum reveals a higher than expected absorbance in the 420–460-nm region, and this may be due to a third minor species involved in catalysis.

Kinetic Competence of Cob(I)alamin. A prerequisite for the establishment of kinetic competence of an intermediate is that the rate constants for both its formation and its decay are as fast as or faster than k_{cat}. The kinetic competence of E·cob(I)alamin as a catalytic intermediate during turnover was evaluated by mixing E·cob(I)alamin with CH_3-H_4folate and homocysteine. E·cob(I)alamin, freshly generated by anaerobic demethylation of E·methylcobalamin (5 μM) by homocysteine (150 μM), was mixed with the substrate mixture (homocysteine, 500 μM; (6*S*)-CH_3-H_4folate, 200 μM), and the formation of E·methylcobalamin was monitored at 390 or 520 nm. Upon addition of the substrate mixture E·cob(I)alamin disappeared very rapidly in a monophasic reaction with an observed rate constant of 250 s^{-1}. This value is 10 times greater than the turnover number. The enzyme returned to the E·cob(I)alamin state at the end of steady-state turnover as expected.

When the complementary experiment was done in which homocysteine was the limiting substrate (homocysteine, 120 μM; (6*S*)-CH_3-H_4folate, 200 μM), the enzyme accumulated as E·methylcobalamin at the end of turnover as shown in Figure 4. From the relative amplitude of changes observed before and after steady state, the proportion of the enzyme present as E·cob(I)alamin in turnover was estimated to be 39%. The UV–visible absorbance spectrum of the enzyme methylated with CH_3-H_4folate was that of E·methylcobalamin in which the 5,6-dimethylbenzimidazole substituent of the corrin ring is coordinated to the cobalt in the so-called "base-on" conformation.

Having demonstrated that E·cob(I)alamin reacts rapidly with CH_3-H_4folate, we next sought to determine the rate of

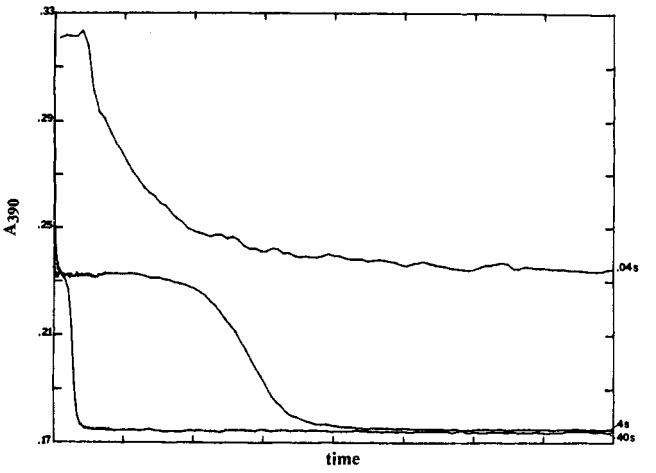


FIGURE 4: Changes in absorbance during turnover starting with the E·cob(I)alamin form of the enzyme with CH_3-H_4folate present in excess over homocysteine. Enzyme in the cob(I)alamin form (5 μM after mixing) was mixed with CH_3-H_4folate (200 μM) and homocysteine (125 μM). When homocysteine was depleted, the enzyme-bound prosthetic group accumulated as methylcobalamin.

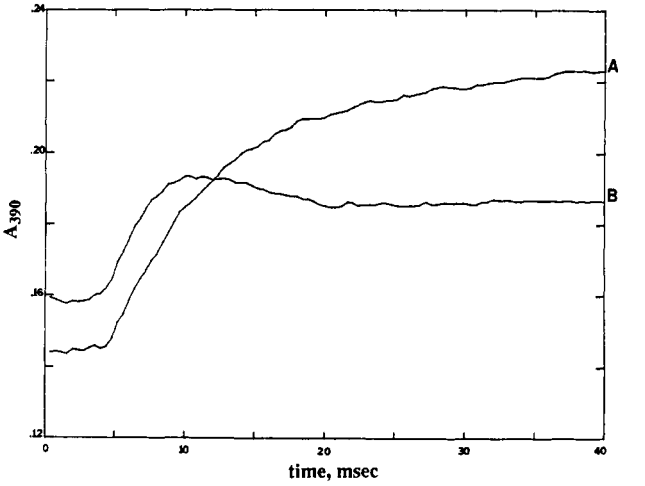


FIGURE 5: Comparison of the initial rate of demethylation of E·methylcobalamin by homocysteine in a binary (A) versus a ternary (B) complex. (A) 5 μM E·methylcobalamin was mixed with 500 μM homocysteine. (B) 5 μM E·methylcobalamin was mixed with 500 μM homocysteine and 200 μM CH_3-H_4folate. The reported optical density has not been corrected for the absorbance due to CH_3-H_4folate and H_4folate.

conversion of E·methylcobalamin to E·cob(I)alamin upon reaction with homocysteine. Methylated methionine synthase (5 μM) was mixed with homocysteine (500 μM) and the reaction was monitored at either 390 or 520 nm. The demethylation reaction consisted of several phases, with approximately 70% of the changes occurring in the fast phase (k = 140 s^{-1}). The observed rate constants associated with the two slower phases were too slow to be important in steady-state turnover. A comparison of the initial rate of demethylation in the binary complex of enzyme and homocysteine (Figure 5, curve A) and in the ternary, turnover mixture (Figure 5, curve B) reveals that the two rates are very comparable and fast. From this it may be concluded that E·cob(I)alamin forms and decays at rates that are sufficiently fast to be relevant in turnover.

Demethylation of Methylcobalamin by H_4folate. The rate constant for the demethylation of E·methylcobalamin by H_4folate was determined by mixing methylated methionine synthase (5 μM) with (6*S*)-H_4folate (50 μM). The reaction was monitored at either 390 or 520 nm. The dealkylation reaction in the binary complex was characterized by several

phases, with ca. 40% of the total change occurring in the fast phase (k = 60 s^{-1}, data not shown). As with the demethylation reaction with homocysteine described above, only the first phase was fast enough to be catalytically significant. Since the enzyme is monomeric, the several phases of the reactions are difficult to explain. It is possible that the enzyme is present in more than one conformational state, with the active conformer being recruited upon binding of ligand. The disparate amplitude changes seen in the different phases with the two dealkylating agents (homocysteine versus H_4folate) argue against the assignment of changes occurring in the slow phases to the presence of multiple preexisting enzyme forms that interconvert only slowly.

Demethylation of Methylcobalamin in the Presence of AdoMet. To ascertain whether AdoMet has access to E·cob(I)alamin formed by demethylation of E·methylcobalamin generated in a binary complex, methylated enzyme (5 μM) was mixed with homocysteine or H_4folate in the presence of AdoMet (70 μM, premixed with the enzyme). The fast phase of demethylation of methylated enzyme by either homocysteine or H_4folate was slowed down in the presence of AdoMet by approximately a factor of 2 (data not shown). These results indicate that in the absence of the second substrate, AdoMet does have access to the enzyme.

Methylation of E·Cob(I)alamin by CH_3-H_4folate in a Binary Complex. To assess the rate of methylation of E·cob(I)alamin by CH_3-H_4folate in a binary complex, methylated methionine synthase (5 μM) was demethylated with H_4folate (18 μM) and mixed with CH_3-H_4folate (200 μM). Absorbance changes were monitored at 390 or 520 nm. A monophasic reaction was seen, with an observed rate constant of 50 s^{-1} (data not shown).

Discussion

Steady-state and pre-steady-state kinetic analyses of the reaction catalyzed by cobalamin-dependent methionine synthase are presented in this paper. Initial velocity studies yielded a set of parallel lines as shown in Figure 1 and suggested either a ping-pong mechanism or an ordered sequential mechanism in which either $K_{dA} \ll K_{mA}$ and/or $K_{mB} \ll$ the lowest concentration of substrate [B] employed in the assays. Product inhibition studies were then undertaken to distinguish between these two possibilities. Since methionine synthase catalyzes an essentially irreversible reaction, the predicted product inhibition patterns for an irreversible ping-pong or ternary mechanism must be compared with the observed patterns. The results (Table II) from the product inhibition analysis are generally consistent with an ordered sequential mechanism in which CH_3-H_4folate binding precedes binding of homocysteine and release of methionine precedes release of H_4folate.

The kinetic constants obtained from the steady-state initial velocity measurements are presented in Table I. Since calculation of the value of K_{dA} requires knowledge of the value for K_{mB} (eq 2) and since the K_m for homocysteine is immeasurably low, the K_d for CH_3-H_4folate could not be determined from these studies. The very low K_m for homocysteine suggests that the enzyme displays synergism in the binding of the two substrates and that the enzyme is always saturated with homocysteine under in vivo conditions.

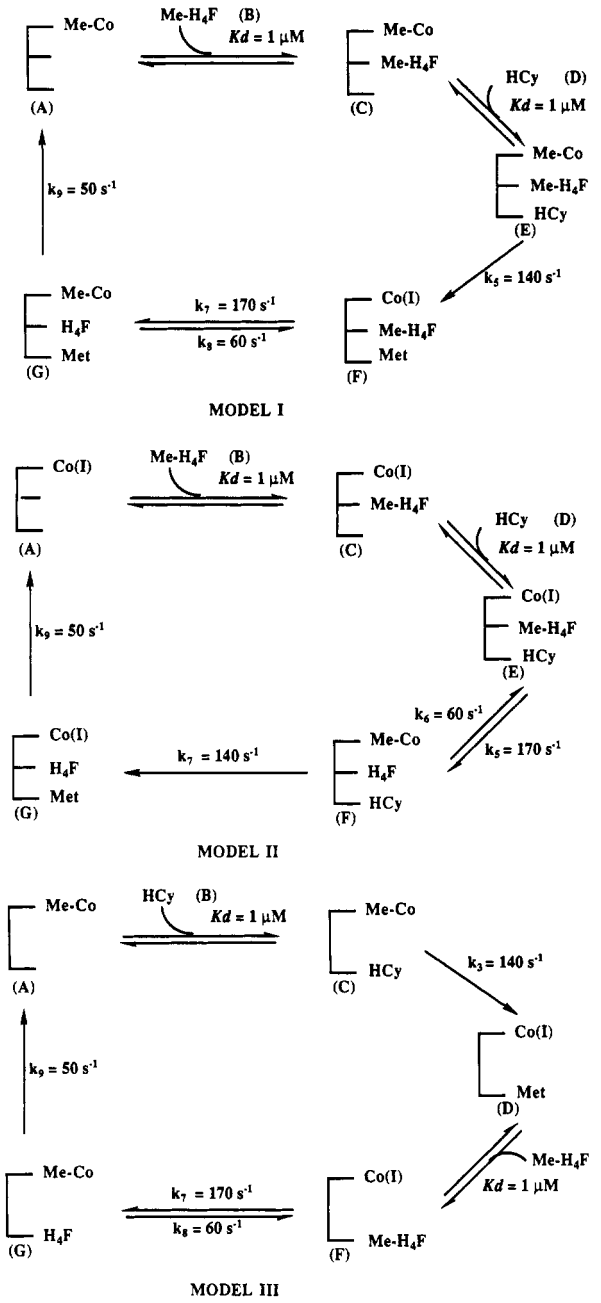
The maximum turnover number for fully active enzyme was always twice as high under the stopped-flow assay conditions as compared to the standard in vitro assay conditions. This discrepancy is presumably related to more rigorously anaerobic conditions in the stopped-flow instrument, which reduce oxidative decay of the labile E·cob(I)alamin intermediate generated during turnover, and suggests that the standard assay routinely underestimates enzyme activity by at least a factor of 2.

For a sequential mechanism, the product inhibition data predict the order of substrate binding, but they provide no information on the nature of the enzyme species to which the substrates bind. The form of the enzyme to which substrates bind will be referred to as "free" enzyme. A previously unanswered question is the identity of the "free" enzyme to which the substrates bind; i.e., is it E·methylcobalamin or E·cob(I)alamin? In order to distinguish between these two possibilities, we simulated the pre-steady-state kinetics under turnover conditions for the two kinetic models shown in Scheme III, employing the rate constants assigned as described below and the known extinction coefficients for E·methylcobalamin (10 500 M^{-1} cm^{-1}) and E·cob(I)alamin (26 300 M^{-1} cm^{-1}) at 390 nm (Banerjee & Matthews, 1990).

Two variants of a minimal kinetic mechanism that accommodate all the data from the steady-state analyses are presented in Scheme III. In model I, the "free" enzyme is in the E·methylcobalamin state, whereas the "free" enzyme in model II is E·cob(I)alamin. The following assumptions were made in the assignment of the rate constants for model I: (1) The measured rate constant for the demethylation of methylcobalamin by homocysteine in the binary complex was 140 s^{-1}. A comparison of the initial rates for the demethylation in binary and ternary complexes showed them to be very comparable as shown in Figure 5 and formed the basis for the assignment of k_5 = 140 s^{-1} in Scheme III. (2) k_8 (60 s^{-1}) represents the measured rate constant for the demethylation of E·methylcobalamin by H_4folate in a binary complex, and, in applying it to the ternary mechanism, it is assumed that in this instance too the demethylation rates are similar in the binary and ternary complexes. (3) When methylated enzyme is mixed with both substrates, the pre-steady-state rate constant observed for demethylation is 250 s^{-1}. This observed rate constant is a complex function and is comprised of the rates of formation and breakdown of E·cob(I)alamin, i.e., $k_{obsd} = k_5 + k_7 - k_8$ (Scheme III, model I). Hence, a preliminary estimate for k_7 was 170 s^{-1}. (4) Assignment of k_9, the product release step, was made from the experiment in which methylation of E·cob(I)alamin (generated by dealkylation with an excess of H_4folate) by CH_3-H_4folate was followed. The reaction proceeded with an observed rate constant of 50 s^{-1} and is considerably slower than the deduced value for k_7 of 170 s^{-1}. Since the demethylating agent, H_4folate, was present in excess, it is reasonable to suppose that the form of the enzyme that was mixed with CH_3-H_4folate had H_4folate bound to it. The observed slow methylation rate might then reflect the rate constant for product (H_4folate) release, k_9. The measured turnover number of 18–20 s^{-1} and the assignment of the other rate constants place constraints on the magnitude of k_9. (5) Binding of CH_3-H_4folate to the enzyme was arbitrarily assigned a value of 1 μM. Increasing this number 250-fold had no effect on the outcome of the simulations (data not shown). (6) Since the K_d for homocysteine is not known, a value of 1 μM was arbitrarily assigned, and the simulations were found to be insensitive in the 0.1–10 μM range. The same set of assumptions were employed for the alternative model. Thus, in model II, k_5 = 170 s^{-1}, k_6 = 60 s^{-1}, k_7 = 140 s^{-1}, and k_9 = 50 s^{-1}. The calculated turnover number under V_{max} conditions with this set of rate constants is 25 s^{-1}, in good agreement with the measured value, and supports the assignment of the various rate constants described above.

The kinetics for the conversion of E·methylcobalamin to E·cob(I)alamin were simulated for both models, i.e., starting

Scheme III: Minimum Kinetic Scheme Consistent with the Steady-State Kinetic Analyses of Methionine Synthase[a]



[a] Models I and II are distinguished by the "free" form of the enzyme, with E·methylcobalamin being the "free" form in model I and E·cob(I)alamin being the form of the enzyme to which substrates bind in model II.

at A in model I and F in model II and using the assigned kinetic constants described above. The simulations along with the observed data are presented in Figure 6 (top) and show that the overshoot in E·cob(I)alamin formation observed in approach to steady state is predicted only for model I. Similarly, conversion of E·cob(I)alamin to E·methylcobalamin was simulated beginning with F in model I and A in model II. Again, the results shown in Figure 6 (bottom) show that only the simulation for model I fits the data. In addition, the two models make very different predictions for the steady-state composition of the enzyme, which is governed by the species that accumulates before the rate-limiting product release step. Hence, model I predicts that 67% of the enzyme will be in the E·methylcobalamin form in steady state, with the remainder (33%) being in the E·cob(I)alamin form. In contrast, model

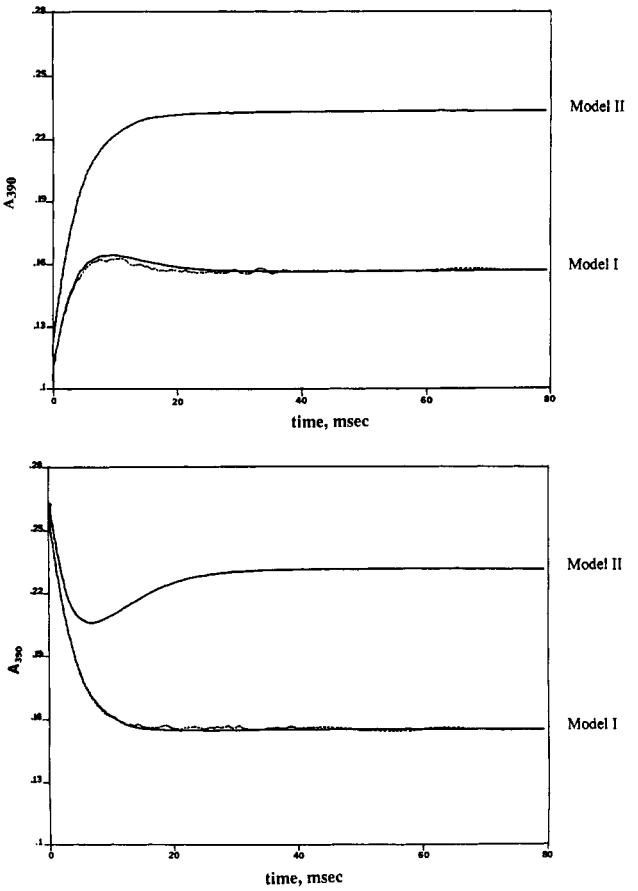


FIGURE 6: Comparison of the observed pre-steady-state kinetics (---) and simulated pre-steady-state kinetics (—) under turnover conditions for models I and II of Scheme III. (Top) Simulated pre-steady-state kinetic trace starting from E·methylcobalamin (data from Figure 2). (Bottom) Simulated pre-steady-state kinetic trace starting from E·cob(I)alamin. The measured values for the rate constants were employed for these simulations. The K_d values for the two substrates and the individual rate constants employed to perform the simulation are described in the text. The observed data were corrected for absorbance at 390 nm due to CH_3-H_4folate and H_4folate.

II predicts the steady-state composition to be 69%:31% in favor of cob(I)alamin. Again, the data fit the predictions made for model I and thus establish the identity of the resting form of the enzyme as E·methylcobalamin.

It should be noted that although the simulations serve to determine the resting form of the enzyme in an ordered sequential mechanism (i.e., models 1 and 2 as depicted in Scheme III), they do not distinguish between a ping-pong and an ordered sequential mechanism. Simulations for a ping-pong mechanism (model III, Scheme III) also give very good fits to the data (not shown).

The assigned rate constants were then adjusted in simulations to obtain values of rate constants that best fit the data for model I. This is necessary since in such a complex mechanism simple analysis of the data does not permit direct extraction of pure rate constants, unless they are very well separated. Individual rate constants were systematically varied while all the others were held constant (data not shown) to determine the influence of each rate constant on the shape of the curve. Larger values for k_5 increased both the amount of E·cob(I)alamin formed in the pre-steady-state burst and the concentration of E·cob(I)alamin in the steady-state. Larger values for k_7, the rate of conversion of E·cob(I)alamin to E·methylcobalamin, predominantly decreased the amount of cob(I)alamin present during steady state. Changing k_8 had a significant effect on the magnitude of the overshoot, which

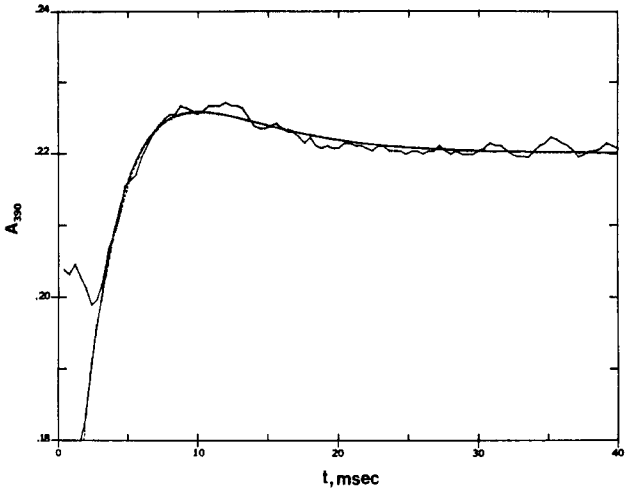


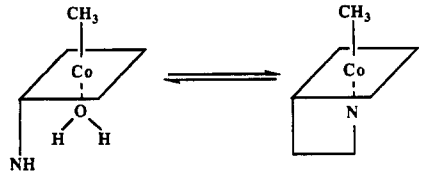
FIGURE 7: Comparison of the simulated best-fit trace (---) and exponential fit (—) to the demethylation rate of E·methylcobalamin under turnover conditions. The experimental data are from Figure 2, and the simulations were performed as described under Discussion. The double-exponential fit to the data was obtained by employing the following parameters for the first and second phases: $\Delta A_1 = 0.567$, $k_1 = 250\ s^{-1}$, $\Delta A_2 = -0.48$, and $k_2 = 225\ s^{-1}$. The rate constants employed to achieve the simulated fit were as follows: $k_5 = 150\ s^{-1}$, $k_7 = 200\ s^{-1}$, $k_8 = 80\ s^{-1}$, $k_9 = 50\ s^{-1}$, and $k_{10} = 0$. K_d values of 1 μM for CH_3-H_4folate and 1 μM for homocysteine were employed to describe the substrate binding steps. The data from Figure 2 have not been corrected for absorbance at 390 nm due to CH_3-H_4folate and H_4folate.

becomes more pronounced as the value for k_8 is decreased. The effects of varying the individual rate constants suggests that the rate constant describing the approach to steady-state is defined predominantly by the relative magnitudes of k_5, k_7, and k_8 with the permissible range for each of the rate constants being $k_5 = 140$–$150\ s^{-1}$, $k_7 = 170$–$200\ s^{-1}$, and $k_8 = 60$–$80\ s^{-1}$. Varying k_9 has little effect on the approach to steady-state, although it profoundly affects the turnover number and thus provides a valuable constraint. Hence, in fitting the simulations to the data, k_9 was not varied, while k_5, k_7, and k_8 were adjusted to yield improved fits. An excellent fit to the data was obtained in the simulation by employing the rate constants $k_5 = 150\ s^{-1}$, $k_7 = 200\ s^{-1}$, $k_8 = 80\ s^{-1}$, $k_9 = 50\ s^{-1}$, and $k_{10} = 0$ and is shown in Figure 7. The values for the individual rate constants obtained by simulations are within experimental error of the measured values. The simulations serve to strengthen the assignment of the various rate constants and corroborate the validity of the described kinetic mechanism.

The calculated turnover number from the simulated data is $25\ s^{-1}$ and is in good agreement with the values of 18–20 s^{-1} obtained from the steady-state measurements under stopped-flow conditions. The chemical conversion steps are relatively fast compared to the turnover number, and the rate-limiting step is associated with product release. Hence, the cobalamin species that accumulates before the product release step, i.e., methylcobalamin, is expected to accumulate during turnover. The ratio of the net rate constants for the methylation and demethylation steps predicts the distribution of the cobalamin species in turnover. The predicted distribution for model I, in which methylcobalamin is the free enzyme, is 33% cob(I)alamin and 67% methylcobalamin, which agrees very well with the observed distribution and provides additional support for the validity of model I.

Given the reactivity of cob(I)alamin, a rationale for a ternary mechanism in turnover becomes clear. E·cob(I)alamin is generated as an intermediate only when the second substrate, CH_3-H_4folate, is bound, a strategy needed to contain the

Scheme IV: Interconversion of Base-On and Base-Off Forms of E·Methylcobalamin[a]



Base-off Methylcobalamin Base-on Methylcobalamin

[a] The base, dimethylbenzimidazole, provides a nitrogen for coordination to the cobalt in the base-on form.

promiscuity of E·cob(I)alamin. This mechanism also explains the lack of access of AdoMet to E·cob(I)alamin generated during turnover, although AdoMet is capable of reaction with photochemically generated E·cob(I)alamin (Banerjee et al., 1990). However, methionine synthase does not have an obligate requirement for ternary complex formation for catalysis, since the enzyme *is* able to catalyze reactions in binary complexes. The kinetics of the half-reactions catalyzed by methionine synthase are, however, complex and multiphasic, with a significant proportion of the reaction being too slow to be relevant in turnover. While the physical basis for this phenomenon is not understood, the observed monophasic kinetics in ternary complexes further bolster the conclusion that the enzyme exhibits a "preferred sequential mechanism" for catalysis.

Our results are consistent with the earlier body of evidence [reviewed by Banerjee and Matthews (1990)] suggesting that the reaction catalyzed by methionine synthase involves an E·methylcobalamin intermediate that suffers nucleophilic displacement at the methyl group by the thiolate homocysteine. The product, E·cob(I)alamin, is then remethylated by a second substrate, CH_3-H_4folate, to regenerate E·methylcobalamin. It is interesting to note that the "free" form of methionine synthase is modified by the transfer of the substrate methyl group from the previous turnover. Another example of a modified "free" enzyme is citrate lyase, which cleaves citrate to oxaloacetate and acetate and requires the participation of a covalent acetyl-enzyme thioester involving the sulfhydryl of the 4-phosphopantetheine cofactor bound to the enzyme. At the end of the catalytic cycle, the enzyme remains acetylated from the previous turnover reaction (Wood et al., 1984).

The spectrum of the enzyme during steady-state turnover (Figure 3) suggests the presence of a third minor component (in addition to E·cob(I)alamin and base-on E·methylcobalamin), which is present only during catalysis. A reasonable candidate with an absorbance in the 420–460-nm range is base-off E·methylcobalamin. In solution, E·methylcobalamin may be in either the base-on or base-off conformations (Scheme IV), with the equilibrium between the two forms being governed by the pK_a of the dimethylbenzimidazole base (4.7; Lexa & Saveant, 1983) and the pH of the solution. The coordination of ligands trans to the methyl ligand of E·methylcobalamin strongly affects the susceptibility of the alkyl–cobalt bond to heterolytic cleavage [reviewed by Matthews et al. (1990)]. Krautler (1987) has found that coordination of the 5,6-dimethylbenzimidazole substituent of the corrin ring to the cobalt stabilizes the CH_3–cobalt bond to heterolytic cleavage by ca. 4.2 kcal/mol. Base-on and base-off species can be distinguished readily by their UV–visible absorption spectra; base-on E·methylcobalamin has a characteristic peak at 520 nm, while the base-off species with water coordinated to the lower axial position of the cobalt has a peak at 460 nm. In addition, base-on alkyl cobalamins are thought

to be more resistant to nucleophilic attack because the bulky ligand in the lower coordination position flexes the corrin ring upward producing a cup around the upper alkyl ligand that impedes access of a nucleophile (Lenhert, 1968). Thus, the conversion of methylcobalamin to a base-off form should facilitate heterolytic cleavage.

Although other workers have provided evidence for the formation of a base-off alkylcobalamin species during turnover (Fujii & Huennekens, 1979; Taylor & Weissbach, 1967; Taylor & Hanna, 1973), in our studies, the spectrum of methylated methionine synthase is clearly that of the six-coordinate base-on species at 25 °C, where a mixture of base-on and base-off E·methylcobalamin would be expected according to Fuji and Huennekens (1979). It is possible that a base-off species is generated transiently in the ternary complex and is rapidly demethylated by homocysteine. Conversion of base-on E·methylcobalamin to E·cob(I)alamin is characterized by an isosbestic point at 435 nm. However, the steady-state absorbance spectrum of the enzyme is marked by an increase in absorbance that extends over the 420–460-nm region and is not explained by the simple conversion of base-on E·methylcobalamin to E·cob(I)alamin. The increased absorbance in this region would be consistent with the formation of a base-off E·methylcobalamin intermediate.

In conclusion, we have demonstrated that the catalytic mechanism of methionine synthase involves a ternary complex of enzyme-bound methylcobalamin, CH_3-H_4folate, and homocysteine and effectively occludes AdoMet as a methyl group donor during turnover. We have shown that both cob(I)alamin and methylcobalamin are kinetically competent for participation in catalysis by methionine synthase. Our data are consistent with a nucleophilic displacement of the methyl group from E·methylcobalamin by homocysteine, followed by remethylation of E·cob(I)alamin by CH_3-H_4folate. In addition, the "free" form of the enzyme has been shown to be E·methylcobalamin. These data suggest that a rational approach for designing a drug to target methionine synthase should incorporate features of both substrates to mimic the ternary complex and/or features that mimic a transition state for an S_N2 displacement.

The reactions catalyzed by cobalamins involved in methyl-transfer reactions have been thought to involve heterolytic fission of the carbon–cobalt bond, in contrast to the more widely studied rearrangement reactions catalyzed by adenosylcobalamin-dependent enzymes that have been shown to involve homolytic cleavage resulting in the generation of free radicals [reviewed by Babior (1988)]. The demonstration of the kinetic competence of E·cob(I)alamin, the nucleophilic acceptor of the methyl group in the transmethylation reaction catalyzed by methionine synthase, provides strong evidence for the involvement of heterolytic chemistry in this cobalamin-dependent methyl-transfer reaction.

Acknowledgments

The critical comments of Dr. Lawrence Schopfer at the University of Michigan and his assistance with running the simulation program are gratefully acknowledged.

Registry No. CH_3-H_4folate, 31690-09-2; cob(I)alamin, 18534-66-2; homocysteine, 6027-13-0; methionine synthase, 37290-90-7.

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