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Synergistic, Random Sequential Binding of Substrates in Cobalamin-Independent Methionine Synthase†

Rebecca E. Taurog,‡ Hieronim Jakubowski,§ and Rowena G. Matthews*,‡,||,⊥

Department of Biological Chemistry, Life Sciences Institute, and Biophysics Research Division, The University of Michigan, Ann Arbor, Michigan 48109-1055, and Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, International Center for Public Health, Newark, New Jersey 07101-1709

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ABSTRACT: Cobalamin-independent methionine synthase (MetE) catalyzes the transfer of the N5-methyl group of methyltetrahydrofolate (CH_3-H_4folate) to the sulfur of homocysteine (Hcy) to form methionine and tetrahydrofolate (H_4folate) as products. This reaction is thought to involve a direct methyl transfer from one substrate to the other, requiring the two substrates to interact in a ternary complex. The crystal structure of a MetE·CH_3-H_4folate binary complex shows that the methyl group is pointing away from the Hcy binding site and is quite distant from the position where the sulfur of Hcy would be, raising the possibility that this binary complex is nonproductive. The CH_3-H_4folate must either rearrange or dissociate before methyl transfer can occur. Therefore, determining the order of substrate binding is of interest. We have used kinetic and equilibrium measurements in addition to isotope trapping experiments to elucidate the kinetic pathway of substrate binding in MetE. These studies demonstrate that both substrate binary complexes are chemically and kinetically competent for methyl transfer and suggest that the conformation observed in the crystal structure is indeed on-pathway. Additionally, the substrates are shown to bind synergistically, with each substrate binding 30-fold more tightly in the presence of the other. Methyl transfer has been determined to be slow compared to ternary complex formation and dissociation. Simulations indicate that nearly all of the enzyme is present as the ternary complex under physiological conditions.

Methionine synthases catalyze the methylation of homocysteine (Hcy)[1] by methyltetrahydrofolate (CH_3-H_4folate), yielding methionine and tetrahydrofolate (H_4folate). *Escherichia coli* contains two genes, *metH* and *metE*, specifying two different methionine synthase enzymes that are differentially expressed (*1*). The MetH protein is cobalamin-dependent methionine synthase, in which the cobalamin (B_{12}) cofactor serves as an intermediary in methyl transfer, accepting a

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* To whom correspondence should be addressed. E-mail: rmatthew@umich.edu. Fax: (734) 763-6492. Phone: (734) 764-9459.

‡ Department of Biological Chemistry, The University of Michigan.

§ University of Medicine and Dentistry of New Jersey.

|| Life Sciences Institute, The University of Michigan.

⊥ Biophysics Research Division, The University of Michigan.

[1] Abbreviations: Hcy, homocysteine; CH_3-H_4folate, (6*S*)-*N*5-methyl-5,6,7,8-tetrahydrofolate; CH_3-$H_4PteGlu_3$, (6*S*)-*N*5-methyl-5,6,7,8-tetrahydropteroyltriglutamate; $^{14}CH_3$-$H_4PteGlu_3$, (6*S*)-*N*5-[*methyl*-^{14}C]-5,6,7,8-tetrahydropteroyltriglutamate; H_4folate, (6*S*)-5,6,7,8-tetrahydrofolate; $H_4PteGlu_3$, (6*S*)-5,6,7,8-tetrahydropteroyltriglutamate; MetE, cobalamin-independent methionine synthase; MetH, cobalamin-dependent methionine synthase; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; TLC, thin-layer chromatography; LB, Luria broth; DEAE, diethylaminoethyl; ITC, isothermal titration calorimetry.

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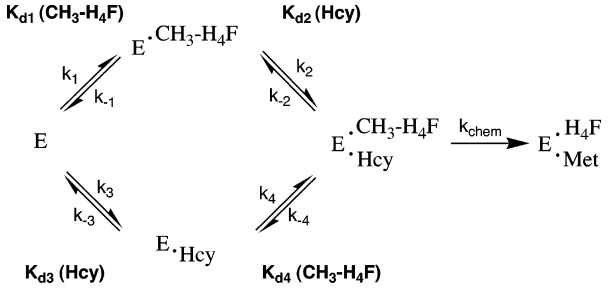
methyl group from CH_3-H_4folate and donating it to Hcy to form methionine. MetH holoenzyme is only expressed under conditions where B_{12} is present in the medium, although the apoenzyme may be constitutively expressed (*2*). Since *E. coli* does not synthesize cobalamin, such conditions are typically encountered during growth of cells in the mammalian gut or under experimental growth conditions where cobalamin has been added to the growth medium. The MetE protein is cobalamin-independent methionine synthase, which does not contain any organic cofactor. This enzyme is believed to catalyze direct transfer of the methyl group between CH_3-H_4folate and Hcy, requiring that both substrates bind to form a ternary complex with MetE. Transcription of *metE* is highest when cells are grown aerobically in the absence of added B_{12} and methionine (*3*) and can constitute 3−5% of the total cellular protein (*4*, *5*).

In addition to the absence of an organic cofactor in MetE, this enzyme differs in its requirements for catalysis from MetH. MetE is the only known folate-dependent enzyme that requires a polyglutamylated folate derivative as a substrate. The monoglutamate CH_3-H_4PteGlu_1 is neither a substrate nor an inhibitor (*5*−*7*). The triglutamate CH_3-H_4PteGlu_3 is typically used as the substrate for in vitro experiments, although the diglutamate substrate also exhibits nearly maximal activity (*8*). MetE catalysis is also stimulated by low concentrations of phosphate and magnesium (*5*). The two enzymes show no detectable sequence homology; however, they do exhibit some similarities. Both enzymes employ a catalytically essential zinc to coordinate the thiolate of the Hcy substrate (*9*, *10*). The thiol of Hcy has a microscopic pK_a of 10.0 (*11*), and coordination to zinc lowers the thiol pK_a and would be expected to increase the reactivity of the substrate at neutral pH (*12*). Both enzymes bind the unprotonated form of CH_3-H_4folate to produce the binary complexes (*12*, *13*), although protonation at N5 is required in order to generate the product H_4folate.

Crystal structures have recently been determined for MetE from *Thermotoga maritima* in complex with each of its two substrates (*14*). The enzyme is a monomer, and the polypeptide folds into two $(\alpha/\beta)_8$ barrels that are positioned in a face-to-face orientation. The catalytically essential zinc ion is bound in the C-terminal barrel by four residues from the protein (*15*, *16*). When Hcy binds to MetE, the δ-sulfur coordinates the zinc and displaces Glu665, the ligand on the opposite side of the zinc. Thus binding of Hcy results in activation of this substrate in the binary complex as the thiol is converted to a thiolate.

In contrast, CH_3-H_4PteGlu_3 appears to be bound in a nonproductive mode in the absence of Hcy. In the crystal structure of the MetE·CH_3-H_4PteGlu_3 binary complex, CH_3-H_4PteGlu_3 interacts with residues from both the N- and C-terminal barrels and is held in a cleft between the two domains. However, the N5-methyl group is approximately 14 Å from the zinc ion and points away from the Zn^{2+}/Hcy site (*14*). It is possible that the observed CH_3-H_4PteGlu_3 binary complex represents a dead-end complex that must dissociate before the reaction can occur. However, the number and types of conserved interactions between the protein and folate suggest that it is biologically relevant. If the binary complex observed in the crystal structure is catalytically competent, then CH_3-H_4PteGlu_3, and perhaps the protein as well, would have to undergo a conformational change to bring the N5-methyl and Hcy sulfur close together for direct group transfer. The structures of the two binary complexes suggest that the order of substrate binding may be important in MetE. Additionally, if protonation of CH_3-H_4folate occurs only in the ternary complex, one must consider the possibility that the proton is derived from Hcy, in which case an ordered substrate binding in which CH_3-H_4PteGlu_3 binds before Hcy would be expected, since Hcy is deprotonated upon binding to MetE.

Scheme 1: MetE Substrate Binding Scheme



In this study we have determined the kinetic constants and equilibrium dissociation constants for the binding of each substrate to MetE to form binary and ternary complexes (Scheme 1). We have shown that both binary complexes are chemically competent and substrate binding is not ordered. We have also demonstrated that the two substrates bind cooperatively. The crystal structures of the two MetE binary complexes suggest a molecular mechanism for this synergy (*14*).

MATERIALS AND METHODS

Materials. Methionine was obtained from ICN (Aurora, OH); dithiothreitol and AG1-X8 resin were purchased from Bio-Rad (Hercules, CA). Methionine sulfoxide was obtained from Aldrich. EcoLite scintillation fluid, potassium phosphate, and all solvents for TLC and HPLC, including HPLC-grade water, were purchased from Fisher Scientific (Fair Lawn, NJ). Hcy thiolactone and all other materials were obtained from Sigma (St. Louis, MO).

Expression and Purification of MetE. MetE was overexpressed in the methionine-requiring *E. coli* K-12 strain GW2531, which lacks endogenous MetE (*17*) and expresses wild-type MetE from pJG816 (*18*). Bacteria from a frozen glycerol stock were streaked onto a LB/agar plate containing 50 or 100 μg/mL ampicillin and allowed to grow for about 24 h. Overnight cultures were inoculated from single colonies and grown in LB medium supplemented with 100 μg/mL ampicillin. Six Fernbach flasks, each with 1 L of LB medium supplemented with 100 μg/mL ampicillin and 0.5 mM zinc sulfate, were inoculated with 1−3 mL of overnight culture in the same medium. Liquid cultures were grown at 37 °C with shaking at 250 rpm for approximately 30 h before harvesting. The protein was purified on a DEAE column as previously described (*18*) with the modifications described in Hondorp et al. (*19*). The protein concentration was ascertained using the molar extinction coefficient $\epsilon_{280} = 157000\ M^{-1}\ cm^{-1}$ (*19*).

Synthesis of CH_3-H_4PteGlu_3 and $^{14}CH_3$-H_4PteGlu_3. (6*S*)-CH_3-H_4PteGlu_3 was synthesized as previously described (*20*) by incubating PteGlu_3 (Schirks Laboratories, Jona, Switzerland) overnight at room temperature with NADPH, formaldehyde, *Lactobacillus casei* dihydrofolate reductase, and pig liver methylenetetrahydrofolate reductase, along with a

reducing system of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The product was purified by FPLC on an anion-exchange column and desalted using SepPak C18 cartridges (Waters, Milford, MA) as previously described (*21*). (6*S*)-[*N*5-*methyl*-^{14}C]CH_3-H_4PteGlu$_3$ ($^{14}CH_3$-H_4PteGlu$_3$), specific radioactivity 24072 dpm/nmol, was synthesized similarly using [^{14}C]formaldehyde (*18*).

Isothermal Titration Calorimetry. An isothermal titration calorimeter (ITC, VP model from Microcal, Northampton, MA) was used to determine the K_d for Hcy in the binary complex with MetE. The binding enthalpies were determined by titrating 1 mM Hcy into 50 μM MetE, both in MetE assay buffer (50 mM Tris, 10 mM potassium phosphate, pH 7.2, 100 μM $MgSO_4$, and 500 μM dithiothreitol) at 25 °C. A titration of Hcy into buffer alone was used as a blank, and the data were subtracted from the protein titration data. Enthalpy data were analyzed using Origin 7.0 (OriginLab Corp., Northampton, MA) and assuming a single binding site, using eq 1 (*22*), where Q is the cumulative heat of binding, n is the stoichiometry of the binding reaction, ΔH is the enthalpy of the reaction, V_0 is the volume of the ITC cell, $[S]_t$ is the total substrate concentration, $[E]_t$ is the total enzyme concentration, and K_a is the equilibrium association constant. The terms ΔH, n, and K_a were optimized to fit the experimental data.

$$Q = \frac{(n[E]_t \Delta H V_0)}{2}\left\{1 + \frac{[S]_t}{n[E]_t} + \frac{1}{nK_a[E]_t} - \sqrt{\left(1 + \frac{[S]_t}{n[E]_t} + \frac{1}{nK_a[E]_t}\right)^2 - \frac{4[S]_t}{n[E]_t}}\right\} \quad (1)$$

Single Turnover Methyl Transfer. A solution of MetE and $^{14}CH_3$-H_4PteGlu$_3$ was preincubated at 25 °C alongside a solution of Hcy. Both solutions contained 50 mM Tris, 10 mM potassium phosphate, pH 7.2, 100 μM $MgSO_4$, and 1 mM dithiothreitol. Reactions were either run and quenched in a quench-flow instrument (KinTek Corp., Austin, TX) or performed on the benchtop and quenched by hand (only time points ≥3 s). In a quench-flow instrument 15 μL of 120 μM MetE/120 μM $^{14}CH_3$-H_4PteGlu$_3$ solution (60 μM each final concentration) was reacted with 15 μL of 2 mM Hcy solution (1 mM final concentration) and quenched with 90 μL of 10% trifluoroacetic acid. For reactions performed on the bench, 8 μL of 75 μM MetE/31 μM $^{14}CH_3$-H_4PteGlu$_3$ solution (60 μM MetE, 25 μM $^{14}CH_3$-H_4PteGlu$_3$ final concentrations) was mixed with 2 μL of 5 mM Hcy (1 mM final concentration) by hand in a microcentrifuge tube. The substrates were allowed to react for varying times before hand-quenching with 90 μL of 10% trifluoroacetic acid. Regardless of the quench method, the trifluoroacetic acid was extracted three times with 200 μL of ethyl ether, the residual acid was neutralized with 100 μL of 1 M Tris, pH 7.0, and the ionic strength of the solution was decreased with 500 μL of water. This mixture was applied to an AG1-X8 anion-exchange column made in a Pasteur pipet (~6.5 cm long) and eluted with 2 mL of water. At neutral pH the highly charged CH_3-H_4PteGlu$_3$ sticks to the column while the zwitterionic amino acids flow through. The eluent was mixed with 10 mL of EcoLite scintillation fluid and counted in a liquid scintillation counter (Beckman LS 6500). The quantity of [*methyl*-^{14}C]Met formed was determined from the amount of radioactivity in the column eluent. The data were fit with eq 2 using KaleidaGraph (Synergy Software, Reading, PA)

$$[P] = [P_\infty](1 - e^{-(kt)}) \quad (2)$$

in which P is [*methyl*-^{14}C]Met. The rate constant was determined as the average of four independent experiments; the error was determined as the standard deviation across experiments.

Preparation of Hcy. Hcy was prepared from L-homocysteine thiolactone by base-catalyzed hydrolysis as previously described (*21*). While treatment with base is estimated to cause ~10% racemization at the Hcy Cα, MetE does not appear to bind or react with D-Hcy. The Hcy concentration was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) as previously described (*23*). The Hcy was aliquoted and stored at −80 °C. Aliquots were only used on the day they were thawed.

Steady-State Assays. The steady-state turnover of MetE was measured using a nonradioactive assay in which the reaction was quenched at various time points and the H_4-PteGlu$_3$ product was acid-treated to form methenyl-H_4-PteGlu$_3$, which was monitored by its absorbance at 350 nm (*21*). Modifications to the published protocol are as follows: assays were run at 25 °C in 400 μL volumes and quenched with 100 μL of acid.

Synthesis and Purification of [^{35}S]Homocysteine Thiolactone. [^{35}S]Homocysteine thiolactone was synthesized from L-[^{35}S]Met as previously described (*24*). Cold L-Met (137.5 nmol) was added to 5.45 mCi of carrier-free [^{35}S]Met (in aqueous solution with 0.1% 2-mercaptoethanol; Amersham, Piscataway, NJ, catalog no. SJ235); one-tenth of the solution was removed to use as a reference, and the rest was split between two microcentrifuge tubes and lyophilized to dryness. The [^{35}S]Met was then dissolved in a total of 300 μL of 57% hydriodic acid with 1% hypophophorous acid and transferred to a 1 mL glass ampule (Wheaton, Millville, NJ; catalog no. 651502). The neck of the ampule was flame-extended but left open, to act as a refluxing tube. A paper towel was inserted into the top of the tube to absorb any escaping material. The solution was allowed to react at 128 °C for 4 h on a heating block and then lyophilized to dryness. The products were resuspended in 333 μL of water, and 30 μL aliquots were stored unpurified at −80 °C. Before use, ~5 × 10^8 dpm (10−30 μL) from the [^{35}S]homocysteine thiolactone synthesis mixture were purified (*24*) on two cellulose TLC plates (cellulose-F on PE sheets, 100 μm; catalog no. 106016, Analtech, Newark, DE) cut into 5 × 6.7 cm strips. In the first dimension, separation was affected by 1-butanol/acetic acid/water (4:1:1). The plate was dried, and separation was performed in the second dimension using 2-propanol/ethyl acetate/water (5:5:1) brought to pH 8 with NH_4OH. The [^{35}S]homocysteine thiolactone was visualized on film, which corresponded to a UV-absorbing spot on the plate. The cellulose was scraped from the plate, and the [^{35}S]-homocysteine thiolactone was extracted with three or four 100 μL aliquots of 1 mM HCl; typical yields were 40%. The concentration of pure [^{35}S]homocysteine thiolactone was determined by counting 1 μL of each extraction aliquot. Aliquots were then stored at −80 °C. All purified [^{35}S]-

homocysteine thiolactone was kept in solution, since lyophilized samples degraded quickly, even at −80 °C.

Synthesis of [^{35}S]Hcy. [^{35}S]Homocysteine thiolactone was hydrolyzed as previously described (*24*) by incubating 0.1−0.8 nmol with 2−4 μL of 0.1 M NaOH at room temperature for 5 min. Any resulting homocystine was reduced by incubating the hydrolysate with 1−2 μL of 100 mM dithiothreitol at room temperature for 3 min. The [^{35}S]Hcy was then neutralized with a volume of 0.1 M HCl equal to that of NaOH used in the first step. Unlabeled Hcy was added to achieve the desired concentration and specific radioactivity. Fresh [^{35}S]Hcy was prepared each day, and the efficiency of the hydrolysis reaction was checked by one-dimensional TLC on 6.7 cm long strips of Analtech cellulose in 1-butanol/acetic acid/water (4:1:1) as described above, alongside a standard of cold homocysteine thiolactone (∼10 nmol), which can be visualized as a UV-absorbing spot on the plate. Formation of homocystine was also easily monitored because it runs very slowly ($R_f = 0.09$) compared to Hcy ($R_f = 0.34$), which runs slightly slower than homocysteine thiolactone ($R_f = 0.48$).

[^{35}S]Hcy Trapping Reactions. [^{35}S]Hcy trapping experiments were performed by preincubating a 10 μL pulse of 250 μM MetE and 75 μM [^{35}S]Hcy (specific radioactivity ∼1 × 10^6 dpm/nmol) alongside a 10 μL chase of 14 mM unlabeled Hcy and 0−1 mM CH_3-H_4PteGlu$_3$ for 5 min at 25 °C. Both solutions contained 50 mM Tris, 10 mM potassium phosphate, pH 7.2, 100 μM $MgSO_4$, and 1 mM dithiothreitol. The chase was mixed with the pulse and incubated for 35 s at 25 °C before the addition of 5 μL of concentrated HCl (12 N) to quench the reaction and convert Hcy to the thiolactone (*25*), which separates more easily than Hcy from Met. This conversion could not be pushed to completion but still reduced the concentration of unlabeled and [^{35}S]-labeled Hcy, which aided in separation of Hcy from Met. To determine how much of the total radiolabel in the mixture could form [^{35}S]Met, the pulse was incubated with 1 mM CH_3-H_4PteGlu$_3$ for 20 min, giving an estimate of 100% conversion. To determine 0% conversion, the quench and chase were added together to the pulse solution.

Samples were spun at 16000*g* in a microcentrifuge at 4 °C for 5 min to pellet the protein, and the same volume of supernatant (18−20 μL) was removed from each sample, lyophilized to dryness, and resuspended in 2 μL of deionized water. The resuspended samples could then be stored at −80 °C. Two different methods, HPLC and TLC, were used to separate the [^{35}S]Hcy thiolactone and [^{35}S]Hcy from [^{35}S]-Met and determine the amount of label in the latter.

One microliter of the trapping products was spotted onto an Analtech cellulose TLC plate cut to 10 × 5 cm and run in two dimensions as described for [^{35}S]homocysteine thiolactone purification. The TLC plates were exposed to a phosphorimaging screen for ∼20 min, and the screen was read on a Amersham Biosciences Typhoon 9410 phosphorimager. The amount of radioactivity in the [^{35}S]Met spot was determined using ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA). A TLC plate with serial dilutions of a known concentration of [^{35}S]Hcy was included on the phosphorimaging screen with each experiment, and the intensity was shown to be linear between 1000 and 40000 dpm. The background intensity was determined from the [^{35}S]Met spot in the 0% control reaction and subtracted from all other samples. The average background was 15% of the maximum intensity in each experiment. The intensity of the spot from the 100% conversion reaction, minus the background, corresponded to the total [^{35}S]Hcy present and able to react in the experiment. The MetE·[^{35}S]Hcy was then determined by normalizing for the concentration of [^{35}S]Hcy calculated to be bound based on the K_d. The data were then plotted as the ratio of [^{35}S-Met]/[MetE·^{35}S-Hcy] versus CH_3-H_4PteGlu$_3$ concentration.

The remaining microliter of trapping product was spiked with unlabeled Met, methionine sulfoxide, and homocysteine thiolactone (100 nmol each) and subjected to HPLC analysis as previously described (*25*). The mixture was diluted with water to a final volume of 20 μL and loaded onto a Beckman C18 column (catalog no. 235329) using a Beckman System Gold HPLC (128 solvent module and 168 diode array detector), which was preequilibrated with 10 mM potassium phosphate, pH 7.4, and 1% acetonitrile. Met and Hcy were eluted isocratically into an in-line radioisotope detector (Beckman 171) set to a maximum sensitivity of 40000 dpm; UV absorption and scintillation data were recorded on an analog chart recorder (Pharmacia Biotech Rec 102). After 10 min the acetonitrile concentration was increased to 10% for 5 min to wash the column and then reequilibrated with the original buffer for 25 min. The peaks of product radioactivity were traced onto graph paper, and the volume underneath the [^{35}S]methionine peak was determined by manual integration. These data were treated in the same way as the TLC phosphorimage intensity data. While the results from the two methods were in agreement, the HPLC method appeared to be slightly more consistent. Data represent the average of six independent experiments.

$^{14}CH_3$-H_4PteGlu$_3$ Trapping Reactions. $^{14}CH_3$-H_4PteGlu$_3$ trapping experiments were performed by preincubating a 10 μL pulse of 100 μM MetE and 25 μM $^{14}CH_3$-H_4PteGlu$_3$ alongside a 100 μL chase of 2.25 mM cold CH_3-H_4PteGlu$_3$ and 0−1 mM Hcy for 5 min at 25 °C. Both solutions contained 50 mM Tris, 10 mM potassium phosphate, pH 7.2, 100 μM $MgSO_4$, and 1 mM dithiothreitol. The chase was mixed with the pulse and incubated for 35 s at 25 °C before the addition of 12 μL of concentrated trifluoroacetic acid to quench the reaction. The trifluoroacetic acid was extracted, and the neutralized mixture was separated on AG1-X8 columns as described above in the Single Turnover Methyl Transfer section. The L-[*methyl*-^{14}C]Met in the eluent was counted in a liquid scintillation counter. As a control for 0% trapping, the quench and chase were added together to the pulse solution.

Error Propagation within the Kinetic Scheme. Unless otherwise noted, errors were determined on the basis of deviations of experimental data from their fits and were propagated by standard methods (*26*). When kinetic constants were added or subtracted, the deviation on the resulting number was determined using eq 3 (*26*).

$$\sigma_C = \sqrt{(\sigma_A)^2 + (\sigma_B)^2} \quad (3)$$

where σ_A is the deviation on the constant A, etc. When kinetic constants were multiplied or divided, the deviation on the resulting number was determined using eq 4 (*26*).

$$\frac{\sigma_C}{C} = \sqrt{(\sigma_A/A)^2 + (\sigma_B/B)^2} \quad (4)$$

RESULTS

To describe the order of substrate binding to MetE, a number of kinetic and equilibrium constants were determined. The K_d for formation of the MetE·CH_3-H_4PteGlu$_3$ binary complex was previously determined by monitoring the UV−visible absorption change of CH_3-H_4PteGlu$_3$ upon binding to MetE (*12*). The fit to the absorption change at 320 nm using eq 5 (*27*) yielded a K_d of 10 ± 1 μM.

$$\Delta A_{320} = \Delta A_{320,max}\{[([E]_t + [S]_t + K_d) - \sqrt{([E]_t + [S]_t + K_d)^2 - 4[E]_t[S]_t}]/2[E]_t\} \quad (5)$$

Determination of the Hcy K_d in the Binary Complex. The K_d for Hcy binding to MetE was determined by isothermal titration calorimetry (ITC). The heat of binding was measured by the calorimeter as Hcy was titrated into MetE. The first injection yields the greatest change in heat because all of the injected Hcy is bound by MetE, but as the MetE sites are filled, the heat of injection decreases (Figure 1a). The integration of the changes in heat gives a sigmoidal binding isotherm, which was fit using a single-site model (Figure 1b). The *n*-value for binding was 0.9, indicating that 90% of the Hcy binding sites were occupied in the experiment. The K_d for Hcy binding to MetE was determined to be 39 ± 3 μM by ITC. In agreement with the ITC data, a K_d of 47 ± 10 μM was obtained by placing [^{35}S]Hcy and MetE in microconcentrators and measuring the difference in the amount of radioactivity found in eluent (free) and retentate (enzyme-bound) after a short spin (data not shown).

Determination of k_{chem} and k_{cat}. The k_{chem} was measured under single turnover conditions by directly monitoring the transfer of a ^{14}C-labeled methyl group from $^{14}CH_3$-H_4PteGlu$_3$ to [*methyl*-^{14}C]methionine. The reaction was quenched with 10% trifluoroacetic acid to precipitate the protein. This quench was chosen in part because the reaction products must be at neutral pH in order to separate the $^{14}CH_3$-H_4PteGlu$_3$ from the [*methyl*-^{14}C]Met by ion exchange, and trifluoroacetic acid is easily extracted with ethyl ether. Only background levels of radioactivity were detected in the ether, indicating that neither the substrate nor the product partition into the organic phase. Once the trifluoroacetic acid was extracted, the reaction products had to be brought to neutral pH with 1 M Tris·HCl, pH 7.0. The ionic strength of the resulting solution was too high to effect good separation, so the products were diluted with water, which led to tight binding of the $^{14}CH_3$-H_4PteGlu$_3$ to the resin, while the [*methyl*-^{14}C]Met eluted in the flow-through. To test that the quench was effective at stopping the reaction quickly, the quench was added at the same time as the initiating substrate, Hcy. This reaction yielded a background of no more than 4% of the total radioactivity in the assay. The background was determined in each experiment and subtracted from the other time points. Approximately 90% of the total radioactivity could be seen in product at the longest time points, although the recovery from reactions performed using the quench-flow apparatus was closer to 75%. Under single turnover conditions in which $^{14}CH_3$-H_4PteGlu$_3$ was limiting, the k_{chem} was determined to be 0.25 ± 0.09 s^{-1} (Figure 2).

The k_{cat} for MetE was ascertained by monitoring H_4PteGlu$_3$ formation under steady-state conditions. After various reaction times, the reaction was quenched with 60% formic acid/5 N HCl, converting the H_4PteGlu$_3$ product to methenyl-H_4-PteGlu$_3$, which was quantitated by its absorbance at 350 nm

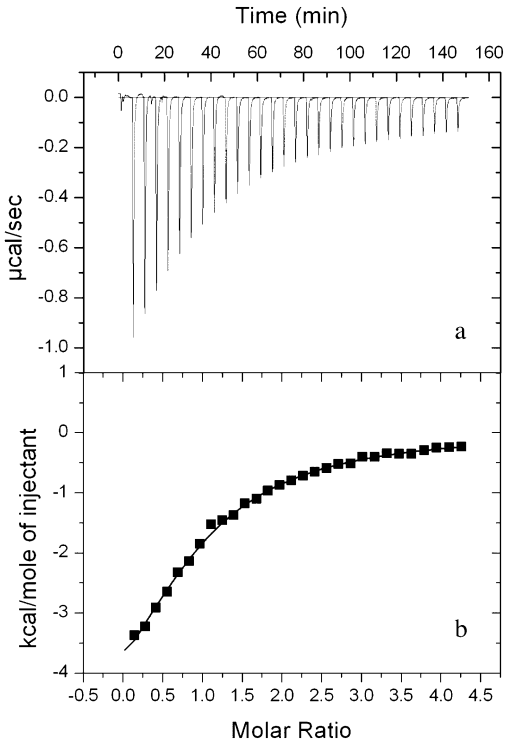
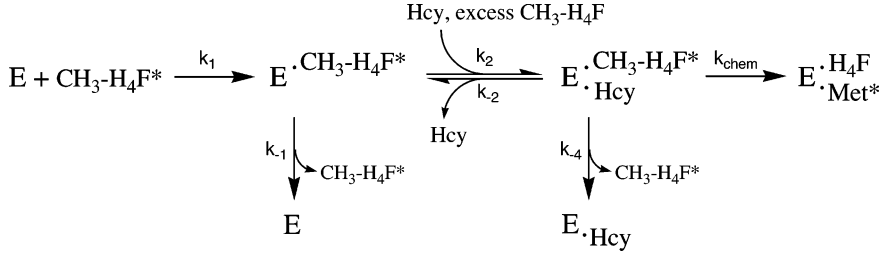


FIGURE 1: Determination of the K_d for Hcy by isothermal titration calorimetry. MetE (50 μM) was titrated with Hcy (1 mM) in MetE assay buffer at 25 °C. (a) Experimental heat change as the ligand is injected after baseline correction. (b) Integrated heats (■) and the best fitting curve using a 1:1 model of binding with a K_d of 39 ± 3 μM.

Scheme 2: Kinetic Scheme for Trapping the Radiolabel from $^{14}CH_3$-H_4PteGlu$_3$ into Met[a]



[a] Dissociation of $^{14}CH_3$-H_4PteGlu$_3$ (abbreviated CH_3-H_4F*) from either the binary or ternary complex is irreversible due to the presence of high concentrations of unlabeled CH_3-H_4PteGlu$_3$. k_{chem} was determined by directly monitoring transfer of a ^{14}C-labeled methyl group (Figure 2) and can be used to determine k_{-4} (see eq 6). The rate constant k_{-1} is determined using eq 8, and k_{-2} is determined in the converse experiment (Scheme 3).

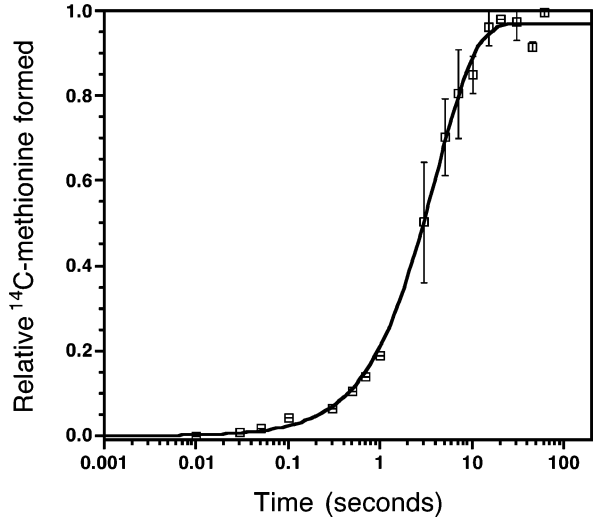


FIGURE 2: Determination of the rate constant for MetE-catalyzed methyl transfer. MetE (60 μM) preincubated with [*methyl*-^{14}C]CH_3-H_4PteGlu$_3$ (60 or 25 μM) was mixed with Hcy (1 mM) in a KinTek quench-flow apparatus or on the bench. The mixture was allowed to react for various time periods and then quenched with 10% trifluoroacetic acid. The [*methyl*-^{14}C]Met was separated from the $^{14}CH_3$-H_4PteGlu$_3$, and product formation was quantitated by liquid scintillation. The data were fit to a single exponential shown by the line; the rate constant, $k_{chem} = 0.25 \pm 0.09$ s^{-1}, was determined as an average from four experiments.

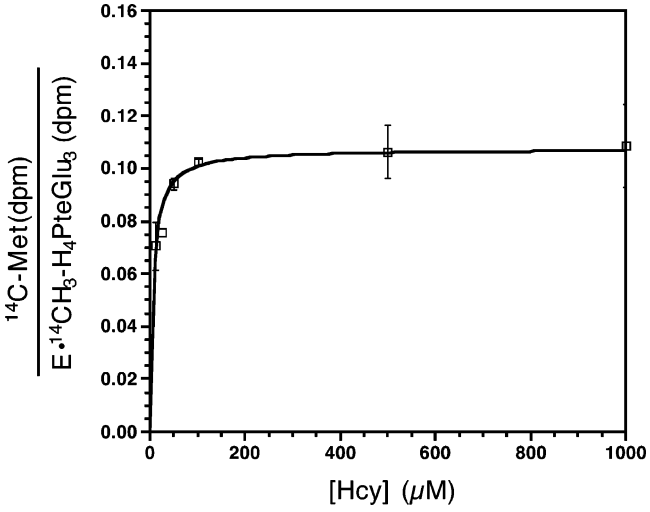


FIGURE 3: Isotope trapping with $^{14}CH_3$-H_4PteGlu$_3$. MetE (60 μM) was preincubated with $^{14}CH_3$-H_4PteGlu$_3$ (25 μM). The MetE·$^{14}CH_3$-H_4PteGlu$_3$ binary complex was mixed with excess CH_3-H_4PteGlu$_3$ (2.25 mM) and varying concentrations of Hcy and allowed to react for 12.6 half-lives before the reaction was quenched with 10% trifluoroacetic acid. The [*methyl*-^{14}C]Met was separated from the $^{14}CH_3$-H_4PteGlu$_3$, and product formation was quantitated by liquid scintillation counting and expressed as the ratio of counts in [*methyl*-^{14}C]Met to MetE·$^{14}CH_3$-H_4PteGlu$_3$ counts in the pulse.

(*28*). Product formation was linear over the time scale measured, and no more than 30% of the substrates had been consumed within this time. The k_{cat} was determined to be 0.12 ± 0.03 s^{-1} at 25 °C.

Determination of Dissociation Rate Constants by Isotope Trapping. Isotope trapping (*29*) was used to investigate both the chemical competence of each MetE binary complex and the kinetics of substrate dissociation (Scheme 2). The methyl transfer catalyzed by MetE is slow and irreversible, precluding the conversion of labeled product back to substrate. Additionally, mixing artifacts are avoided because the mixing time is relatively fast compared to chemistry. If mixing were slow compared to chemistry, more labeled product would be observed because the effective specific radioactivity of the labeled substrate would be higher before mixing was complete, presenting more opportunity for labeled substrate to rebind to free enzyme. Trapping experiments for both MetE substrates were performed with two different ratios of enzyme:labeled substrate, thus changing the amount of free enzyme available. If mixing artifacts were occurring, an increase in the free enzyme concentration would increase the ratio of label observed in the product. However, the data from the two sets of conditions agreed completely.

MetE and $^{14}CH_3$-H_4PteGlu$_3$ were preincubated under conditions where the majority of the radiolabel was bound in the MetE·$^{14}CH_3$-H_4PteGlu$_3$ binary complex. The binary complex was reacted with Hcy and excess unlabeled CH_3-H_4PteGlu$_3$ such that if the $^{14}CH_3$-H_4PteGlu$_3$ dissociated from the enzyme before the ^{14}C-labeled methyl was transferred, it would be replaced by unlabeled substrate. The reaction was run at multiple Hcy concentrations, the [*methyl*-^{14}C]Met was separated from the$^{14}CH_3$-H_4PteGlu$_3$, and the ratio of [*methyl*-^{14}C]Met produced to the initial concentration of E·$^{14}CH_3$-H_4PteGlu$_3$ was determined (Figure 3). A maximum of 10.7 ± 0.3% of the original E·$^{14}CH_3$-H_4PteGlu$_3$ in the reaction was found in methionine. This maximum is dependent only on the partitioning between dissociation from the ternary complex and formation of product, described by eq 6 (*29*), from which the rate constant describing the loss of $^{14}CH_3$-H_4PteGlu$_3$ from the ternary complex, k_{-4}, can be determined.

$$\frac{[\textit{methyl}\text{-}^{14}\text{C}]\text{Met (dpm)}}{\text{E}\cdot^{14}\text{CH}_3\text{-H}_4\text{PteGlu}_3\text{ (dpm)}} = \frac{k_{chem}}{k_{chem} + k_{-4}} \quad (6)$$

The $K_{1/2}$ for Hcy in the trapping reaction was 7 ± 1 μM. By definition, when [Hcy] was equal to the $K_{1/2}$, the amount of label found in product was half-maximal, and the label was partitioning equally between dissociation from the binary complex and all possible paths from the ternary complex. We reasoned that since k_2', the net rate constant for k_2 (eq 7) (*30*), describes the flux to the ternary complex, then k_{-1} should be equivalent to k_2' multiplied by the $K_{1/2}$. This relationship is described in eq 8 , in which k_2' is written in

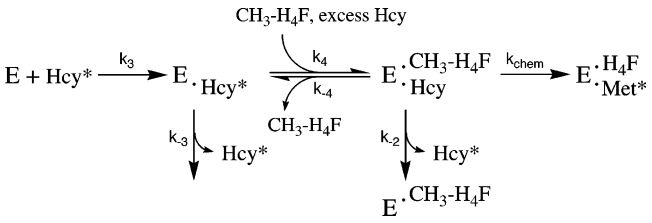
$$k_2' = k_2\left(\frac{k_{chem} + k_{-4}}{k_{-2} + k_{chem} + k_{-4}}\right) \quad (7)$$

$$k_{-1} = k_2 K_{1/2}\left(\frac{k_{chem} + k_{-4}}{k_{-2} + k_{chem} + k_{-4}}\right) \quad (8)$$

expanded form. Thus, k_{-1} can be determined if k_2 and k_{-2} are known.

The converse experiment was performed in which 75 μM [^{35}S]Hcy was preincubated with 250 μM MetE and chased with excess unlabeled Hcy and various concentrations of CH_3-H_4PteGlu$_3$ (Scheme 3 and Figure 4). Up to 14 ± 2% of the radiolabel in the original E·[^{35}S]Hcy complex could be trapped in [^{35}S]Met. However, the $K_{1/2}$ for CH_3-H_4PteGlu$_3$ was 230 ± 90 μM, indicating that high concentrations of CH_3-H_4PteGlu$_3$ were required for maximum trapping. Concerns about the very high concentrations of MetE used in

Scheme 3: Kinetic Scheme for Trapping the Radiolabel from [^{35}S]Hcy into Met[a]



[a] Dissociation of [^{35}S]Hcy from either the binary or ternary complex is irreversible due to the presence of high concentrations of unlabeled Hcy. Rate constants k_{-2} and k_{-3} can be determined from k_{chem}, k_{-4}, and k_4 (see eqs 9 and 10) which were determined in other experiments.

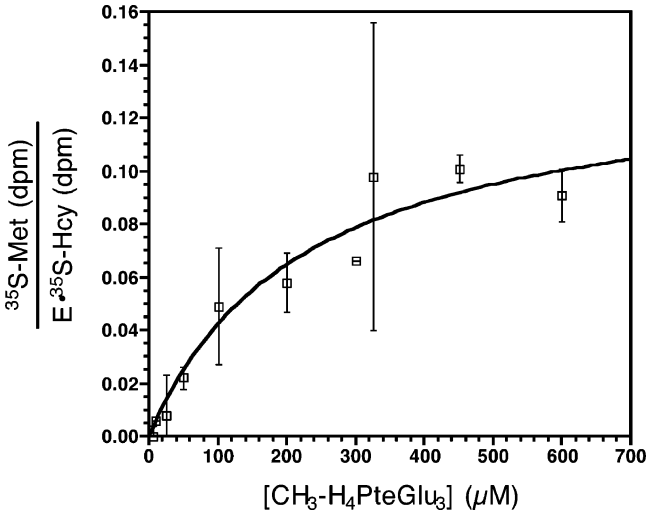


FIGURE 4: Isotope trapping with [^{35}S]Hcy. MetE (250 μM) was preincubated with [^{35}S]Hcy (75 μM). The MetE·[^{35}S]Hcy binary complex was mixed with excess unlabeled Hcy (14 mM) and varying concentrations of CH_3-H_4PteGlu$_3$ and allowed to react for 12.6 half-lives before the reaction was quenched with concentrated HCl. The [^{35}S]Met was separated from the [^{35}S]Hcy and quantitated by reverse-phase HPLC with in-line liquid scintillation counting or by 2D TLC and phosphorimaging. Data are expressed as the ratio of counts of [^{35}S]Met to MetE·[^{35}S]Hcy counts in the pulse.

this experiment led to repetition with 60 μM MetE and 25 μM [^{35}S]Hcy in the pulse; however, this change did not affect the results.

Equations analogous to eqs 6 and 8 permitted determination of k_{-2} (eq 9) and k_{-3} (eq 10), respectively. All calculated rate constants are shown in Table 1.

$$\frac{[^{35}\text{S}]\text{Met (dpm)}}{\text{E}\cdot[^{35}\text{S}]\text{Hcy (dpm)}} = \frac{k_{chem}}{k_{chem} + k_{-2}} \quad (9)$$

$$k_{-3} = k_4 K_{1/2}\left(\frac{k_{chem} + k_{-2}}{k_{-2} + k_{chem} + k_{-4}}\right) \quad (10)$$

Determination of K_d Values for Ternary Complex Formation. When the MetE·CH_3-H_4PteGlu$_3$ binary complex is reacted with Hcy, the absorbance at 324 nm quickly decreases [see Figure 3 in the accompanying paper (*35*)]. The amplitude of this absorbance decrease shows a saturable dependence on Hcy [Figure 5c in the accompanying paper (*35*)]. These absorbance data fit nicely to a quadratic binding equation with a K_d for Hcy of 1.4 $\pm$ 0.5 μM. This K_d appeared to describe the binding of Hcy to the E·CH_3-H_4-PteGlu$_3$ complex to form the ternary complex. The converse experiment in which the initial complex is E·Hcy and binding of CH_3-H_4PteGlu$_3$ initiates the absorbance decrease yielded a K_d of 0.3 $\pm$ 0.1 μM.

Construction of a Kinetic Scheme. To complete the kinetic scheme for MetE, the K_d values for all four binding steps had to be known. The K_d values derived from the substrate dependence of the stopped-flow absorbance change appeared to describe the dissociation of the ternary complex. However, to validate this assumption, three K_d values were used to determine the fourth, since the thermodynamics of the conversion from free enzyme and free substrates to the ternary complex are independent of the path by which the conversion occurs. Of the two K_d values determined using the stopped-flow absorbance changes, the K_d for Hcy binding to E·CH_3-H_4PteGlu$_3$ was chosen as the "known" because it was better defined, due to greater deviation from infinitely tight binding. From this method a K_d of 0.4 $\pm$ 0.2 μM was calculated for the binding of CH_3-H_4PteGlu$_3$ to E·Hcy, which agreed well with the experimentally determined K_d of 0.3 $\pm$ 0.1 μM from the stopped-flow absorbance decrease.

The isotope trapping experiment provided the rate constants for substrate dissociation from both binary and ternary complexes. When combined with the K_d values for these binding events, the bimolecular rate constants for association could be calculated, assuming a one-step binding mechanism. The complete set of equilibrium dissociation constants and rate constants for progression from free enzyme and free substrates to the ternary complex is listed in Table 1.

DISCUSSION

We have directly determined the rate constants and equilibrium binding constants that govern the synthesis of methionine catalyzed by MetE. These constants were used to formulate a kinetic scheme that describes the steps in substrate binding. We have also used isotope trapping to show that both MetE·substrate binary complexes are chemically competent for turnover and to determine the rate constants for formation and dissociation of the binary and ternary complexes shown in Scheme 1.

The meaning of the rate constants determined by isotope trapping must be considered in the context of the kinetics of the absorbance changes observed during a single turnover (see ref *35*). Protonation of CH_3-H_4PteGlu$_3$ in the ternary complex appears to occur in at least two steps, based on the biphasicity of the stopped-flow absorbance changes. These observations suggest that there is more than one ternary complex and that CH_3-H_4PteGlu$_3$ is protonated to differing extents in these forms. The ternary complexes may interconvert, and it is possible that substrates dissociate from more than one form. Therefore, the rate constants for substrate dissociation determined by isotope trapping may actually represent apparent rate constants, which include the microscopic rate constants for interconversion between ternary complexes, product formation, and substrate dissociation from each ternary complex.

Some of the rate constants were calculated from a number of experimentally determined constants, which lead to large errors. The major source of this uncertainty was the [^{35}S]-Hcy isotope trapping experiment due to the challenge of separating [^{35}S]Met from high concentrations of [^{35}S]Hcy. Despite the considerable uncertainties on some rate constants, a number of conclusions can reasonably be drawn.

Table 1: Rate Constants and Equilibrium Constants for Substrate Binding[a]

K_{d1} (E + CH_3-H_4PteGlu$_3$) (μM)	10 ± 1	k_1 ($\mu M^{-1} s^{-1}$)	0.5 ± 0.3
		k_{-1} (s^{-1})	5 ± 3
K_{d2} (E·CH_3-H_4PteGlu$_3$ + Hcy) (μM)	1.4 ± 0.5	k_2 ($\mu M^{-1} s^{-1}$)	1.1 ± 0.6
		k_{-2} (s^{-1})	1.5 ± 0.6
K_{d3} (E + Hcy) (μM)	39 ± 3	k_3 ($\mu M^{-1} s^{-1}$)	16 ± 14
		k_{-3} (s^{-1})	600 ± 500
K_{d4} (E·Hcy + CH_3-H_4PteGlu$_3$) (μM)	0.4 ± 0.2	k_4 ($\mu M^{-1} s^{-1}$)	6 ± 4
		k_{-4} (s^{-1})	2.1 ± 0.8

[a] Errors are given as standard deviations from the mean.

The most striking finding of this study is the synergistic binding of the two substrates. Binding of either substrate appears to increase the affinity for the other ~30-fold. This finding is based on the K_d values for the binary and ternary complexes, which were all measured directly. The molecular basis of this cooperation might be attributable to differences between the substrate-free MetE structure and the structures of the two binary complexes (*14*). In the substrate-free enzyme Met468 (numbering is from *T. maritima*; this residue is 490 in the *E. coli* MetE) is exposed to solvent and pointing away from the zinc, while Trp539 (561 in *E. coli*) occupies two conformations, one of which precludes CH_3-H_4PteGlu$_3$ binding. When CH_3-H_4PteGlu$_3$ binds to MetE, Trp539 becomes ordered in a single conformation and stacks against the pterin ring. In this orientation Trp539 provides hydrophobic contacts for Met468, which swings in and adopts the conformation in which it interacts with the Hcy sulfur when Hcy is bound. Crystal structures of the Hcy binary complex solved at pH 5.2 show that binding of Hcy also triggers these changes, which organize the active site to favor CH_3-H_4PteGlu$_3$ binding. The reader is directed to the original publication of this work (*14*), in which a graphical representation of these changes is presented in Figure 6. However, in crystals grown at pH 8, Trp539 does not appear to assume the CH_3-H_4PteGlu$_3$ binding conformation upon formation of the Hcy binary complex (R. Pejchal and M. L. Ludwig, personal communication). While the causes of these molecular movements may not be simple, there does appear to be cross-talk between the substrate binding sites.

These structural rearrangements are observed in the MetE·CH_3-H_4PteGlu$_3$ binary complex in which the *N*5-methyl points away from the zinc and is unprotonated, reinforcing the conclusion that this complex is indeed biologically relevant. This conclusion is also supported by the observation that ^{14}C from a MetE·$^{14}CH_3$-H_4PteGlu$_3$ binary complex can be trapped into [*methyl*-^{14}C]methionine and the finding in the accompanying paper (*35*) that CH_3-H_4PteGlu$_3$ appears to bind to MetE such that it is initially unprotonated, even in the presence of Hcy. The fact that both substrate binary complexes are chemically competent indicates that there is no required order of substrate binding. However, the possibility that there are multiple binary complexes, only some of which are competent, must be noted. The small percentage of labeled substrate in either binary complex that is actually converted to product is also striking, indicating that the MetE ternary complex has a low commitment to catalysis and is much more likely to dissociate than react. This is a consequence of the very small k_{chem} relative to the rate constants for substrate dissociation.

The very fast dissociation of Hcy from the binary complex is surprising. The error for k_{-3} is quite large, but within the error, the dissociation appears to be at least an order of magnitude faster than other rate constants for dissociation. These data are supported by the observation that when a MetE·[^{35}S]Hcy binary complex was applied to a P6 Biospin size exclusion column, only 0.2% of [^{35}S]Hcy coeluted with MetE, under conditions where 50% of the [^{35}S]Hcy should have been bound, based on a K_d of 39 μM, suggesting that the binary complex is not stable.

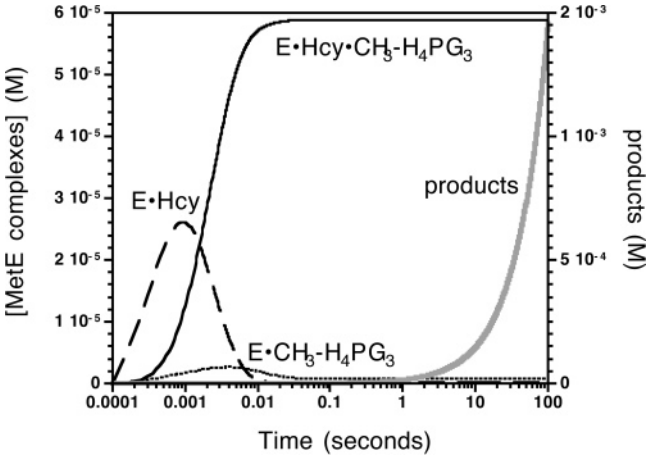


FIGURE 5: Simulation of the MetE reaction under cellular conditions. The MetE reaction was simulated in Berkeley Madonna using the rate constants for binding from Table 1. The concentrations of MetE (60 μM), CH_3-H_4PteGlu$_3$ (130 μM), and Hcy (100 μM) used were based on measurements of their intracellular concentrations in *E. coli*. The left axis indicates the concentrations of E·Hcy (dashed line), E·CH_3-H_4PteGlu$_3$ (dotted line), and E·Hcy·CH_3-H_4PteGlu$_3$ (solid black line). The right axis indicates the concentrations of the products (thick gray line).

The rate constant for chemistry, k_{chem}, is about twice as fast as the k_{cat} determined under the same conditions. This indicates that a step after methyl transfer, such as product release, is partially rate limiting. Experiments described in the accompanying paper (*35*) demonstrate that H_4PteGlu$_3$ probably remains bound to MetE after the reaction and that Hcy can bind to form an E·H_4PteGlu$_3$·Hcy "mixed" ternary complex. It is possible that release of H_4PteGlu$_3$ from either the binary complex or the mixed ternary complex is another partially rate-limiting step.

Using the cellular concentrations of the reactants, we can model the physiological reaction using the simulation program Berkeley Madonna (*31*). The cellular concentration of MetE is ca. 60 μM when *E. coli* K-12 is grown under aerobic conditions in minimal medium (*19*), and the Hcy concentration has been measured to be 0.1 mM (*32*). During exponential growth of *E. coli*, folate accumulates as H_4folate derivatives (*33*) that are mostly triglutamates (*34*). The total concentration of reduced folates has been shown to be ~270

μM (150 ng/10^9 cells) (*33*). If it is assumed that half of the H_4folates are in the *N*5-methyl form, the intracellular concentration of CH_3-$H_4PteGlu_3$ is about 130 μM. Under these conditions the flux through the pathway appears to favor the E·Hcy binary complex over E·CH_3-$H_4PteGlu_3$ (Figure 5), because the E·Hcy binary complex comes to equilibrium very rapidly, due to the fast association and dissociation rate constants. Once the E·Hcy complex is formed, it competes with free MetE for CH_3-$H_4PteGlu_3$, and due to the lower K_d for formation of the ternary complex than the binary complex, very little E·CH_3-$H_4PteGlu_3$ ever builds up. Simulation of MetE kinetics also suggests that the ternary complex accumulates much faster than methyl transfer occurs, such that under physiological conditions nearly all of the enzyme is in the ternary complex. Therefore, this truly is a rapid equilibrium, random-sequential bireactant reaction.

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