Reactivation of methionine synthase from *Thermotoga maritima* (TM0268) requires the downstream gene product TM0269

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Abstract

The crystal structure of the *Thermotoga maritima* gene product TM0269, determined as part of genome-wide structural coverage of *T. maritima* by the Joint Center for Structural Genomics, revealed structural homology with the fourth module of the cobalamin-dependent methionine synthase (MetH) from *Escherichia coli*, despite the lack of significant sequence homology. The gene specifying TM0269 lies in close proximity to another gene, TM0268, which shows sequence homology with the first three modules of *E. coli* MetH. The fourth module of *E. coli* MetH is required for reductive remethylation of the cob(II)alamin form of the cofactor and binds the methyl donor for this reactivation, *S*-adenosylmethionine (AdoMet). Measurements of the rates of methionine formation in the presence and absence of TM0269 and AdoMet demonstrate that both TM0269 and AdoMet are required for reactivation of the inactive cob(II)alamin form of TM0268. These activity measurements confirm the structure-based assignment of the function of the TM0269 gene product. In the presence of TM0269, AdoMet, and reductants, the measured activity of *T. maritima* MetH is maximal near 80°C, where the specific activity of the purified protein is ~15% of that of *E. coli* methionine synthase (MetH) at 37°C. Comparisons of the structures and sequences of TM0269 and the reactivation domain of *E. coli* MetH suggest that AdoMet may be bound somewhat differently by the homologous proteins. However, the conformation of a hairpin that is critical for cobalamin binding in *E. coli* MetH, which constitutes an essential structural element, is retained in the *T. maritima* reactivation protein despite striking divergence of the sequences.

Keywords: structural genomics; adenosylmethionine; cobalamin; structure and function

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Abbreviations: AdoMet, *S*-adenosylmethionine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Hcy, homocysteine; CH₃-H₄folate, 5-methyltetrahydrofolate.

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Methionine synthases catalyze the formation of methionine from homocysteine (Hcy) and methyltetrahydrofolate (CH₃-H₄folate). Two families of methionine synthases are known: the B₁₂-dependent enzymes that use the cobalamin cofactor as an intermediate methyl carrier (Scheme 1), and the B₁₂-independent enzymes that catalyze direct methyl transfer from CH₃-H₄folate to Hcy. The best characterized B₁₂-dependent methionine synthase (MetH)

Scheme 1. The catalytic cycle of MetH (red) and the reactions that deactivate and reactivate the enzyme from *E. coli* (green). In methionine synthesis, cobalamin serves as an intermediate methyl carrier, receiving a methyl group from CH₃-H₄folate and, in turn, donating the group to Hcy. Inactivation by reaction with oxygen generates the cob(II)alamin form, which must be reduced and then remethylated by AdoMet in order to return to the catalytic cycle.

is from *Escherichia coli*, which comprises four structural and functional modules (Banerjee et al. 1989; Drummond et al. 1993; Goulding et al. 1997; Ludwig and Matthews 1997; Matthews 1999). The two N-terminal modules bind the substrates Hcy and CH₃-H₄folate, respectively (Goulding et al. 1997) and the third module carries the essential B₁₂ prosthetic group (Banerjee et al. 1989). The C-terminal module binds AdoMet and is required for the reductive reactivation reaction that rescues the enzyme from its inactive cob(II)-alamin form (Scheme 1; Drummond et al. 1993; Jarrett et al. 1998). This four-module arrangement is prevalent in nature.

Thermotoga maritima expresses MetH as two separate polypeptides, as first suggested by alignments with other MetH enzymes; the gene product TM0268 (residues 1–768) appears to include the three catalytic and B₁₂-binding modules of MetH. However, the homolog of the fourth module of *E. coli* MetH could not be identified by sequence homology, but the crystal structure of TM0269, determined as part of genome-wide structural coverage of *T. maritima* (PDB accession number 1J6R), exhibited a fold very similar to the core of the unique reactivation module of *E. coli* MetH (Dixon et al. 1996). Although TM0269 and the reactivation domain of *E. coli* MetH are structural analogs, implying that TM0269 binds AdoMet and is essential for reactivation of the cob(II)alamin form of TM0268, the functional role of TM0269 has not been directly examined. Here, we report the characterization and functional properties of TM0268 and TM0269 and

demonstrate that TM0269 supports reactivation of the cob(II)alamin form of TM0268.

Formation of the inactive cob(II)alamin form of MetH and its conversion to the active methylcobalamin species have previously been examined in detail with *E. coli* MetH. Inactivation results from reaction with oxygen of the cob(I)alamin species that is formed upon methylation of Hcy and occurs about once in every 2000 turnovers (Drummond et al. 1993). Reactivation proceeds via intermediate formation of cob(I)alamin, which is generated by electron transfer from flavodoxin (Jarrett et al. 1998). The cob(I)alamin form is then remethylated by AdoMet in a step that requires the C-terminal activation domain (Drummond et al. 1993). Reduction to cob(I)-alamin is thermodynamically disfavored, but the overall reaction is driven by the favorable free energy of methyl transfer from AdoMet (Banerjee et al. 1990). For in vitro assays of reactivation, the physiological reducing system, NADPH, ferredoxin(flavodoxin)-NADP⁺ oxidoreductase, and flavodoxin, can be replaced by dithiothreitol as a source of reducing equivalents and by hydroxocobalamin as a catalyst (Taylor and Weissbach 1967; Jarrett et al. 1997). The inactive cob(II)alamin enzyme can also be reduced in an electrochemical cell and remethylated by AdoMet in the presence of a functional reactivation module (Jarrett et al. 1997). Sequences coding for flavodoxin are not found in the *T. maritima* genome; the alternative physiological reductant of cob(II)alamin MetH in this organism has yet not been identified. Our studies of the reactivation of *T. maritima* MetH have, therefore, used these chemical and electrochemical reducing systems.

Results

TM0268 is expressed as a cobalamin-free apo-protein that can be reconstituted to an active species by addition of cobalamin

Histidine-tagged versions of the TM0268 and TM0269 proteins have been expressed singly and together in *E. coli* and have been purified from cell lysates by heat treatment and subsequent separation using Zn-NTA chromatography. Exploration of expression in a variety of hosts and media failed to elicit conditions that would support incorporation of cobalamin during growth of the host cells. However, we were successful in reconstituting the purified TM0268 apo-protein by incubation with methylcobalamin at 60°C.

The visible spectrum confirms that the reconstituted protein remains in the methylcobalamin form (Fig. 1). Measurements of methylcobalamin content based on absorbance at 536 nm, combined with determination of the protein concentration using 5,5'-dithiobis(2-nitrobenzoic

Figure 1. (Solid line) The visible absorbance spectrum of TM0268 reconstituted with methylcobalamin. (Dashed line) For comparison, the spectrum of *E. coli* MetH in the methylcobalamin form.

acid) (DTNB) titration, indicate a 1:1 stoichiometry of protein and cobalamin. This form of the protein can be stored at -80°C for prolonged periods without loss of activity or spectral alterations. As described below, the reconstituted protein catalyzes the synthesis of methionine from Hcy in the presence of TM0269 and AdoMet.

*Maximum activity of Tm MetH (TM0268) was observed at 80°C with a turnover number $\sim 15\%$ that of the *E. coli* enzyme at 37°C*

To determine the temperature dependence of turnover of Tm MetH in vitro, H₄-folate production was measured (Jarrett et al. 1997) in assays initiated by addition of CH₃-H₄folate and including 1:1 (molar) mixtures of the methylcobalamin form of TM0268 and TM0269 along with Hcy, AdoMet, and the chemical reducing system—dithiothreitol and hydroxocobalamin. Use of this protocol allows both deactivation by reaction with Hcy and reactivation (in the presence of AdoMet, TM0269, and the chemical reducing system) during temperature equilibration. Under these assay conditions, 1:1 ratios of the two protein components yielded maximum rates of product formation (data not shown).

The maximum formation of product observed after 2 min occurs between 75°C and 80°C , where the specific activity is $\sim 3 \mu\text{mol}/\text{min}/\text{mg}$ (Fig. 2). For comparison, the specific activity of the *E. coli* enzyme at 37°C in the presence of the complete reactivation system is $\sim 15 \mu\text{mol}/\text{min}/\text{mg}$, corresponding to a turnover number of 34 sec^{-1} . Adjusting for the relative molecular weights, the maximum observed activity of the *T. maritima* enzyme is 5 turnovers/sec, or 15% that of the *E. coli*

enzyme. The optimum growth temperature for *T. maritima* is reported to be 80°C (Daniel and Danson 2001). The decrease in activity at higher temperatures may result not only from instability of the enzyme but also from destruction of the folate substrate at temperatures $>80^{\circ}\text{C}$ (Indrawati et al. 2005).

TM0269 and AdoMet are required to activate the cob(II)alamin form of TM0268 and to maintain the activity of TM0268

The methylcobalamin form of TM0268 isolated after reconstitution was reacted with Hcy to convert to the inactive cob(II)alamin species as described in Materials and Methods. Figure 3 shows product formation as a function of time in assays that were initiated by addition of cob(II)alamin TM0268 in the presence and absence of TM0269. In the absence of TM0269, with or without the addition of AdoMet, the enzyme displays significant, but low, activity (~ 0.07 turnovers/sec or 4 turnovers/min). Similar rates of product formation are also seen in the absence of AdoMet when TM0269 is present. We attribute this slow product formation to a fraction of TM0268 that was not demethylated under the conditions used for reaction of the methylcobalamin enzyme with Hcy. In contrast, in the complete system, product formation proceeds linearly at ~ 20 turnovers/min at this suboptimal temperature. This turnover is substantially lower than that measured at 65°C for enzyme

Figure 2. Specific activity of *T. maritima* methionine synthase as a function of temperature. Assay mixtures included Hcy (saturating), AdoMet, the chemical reducing system, and 1:1 mixtures of TM0268 and TM0269. After temperature equilibration for 2 min, turnover was initiated by addition of CH₃-H₄folate. The amount of product formed was measured in samples quenched at 2 min. (See Materials and Methods for additional details.)

Figure 3. Reactivation of activity of TM0268 requires TM0269 and AdoMet. TM0268 in the cob(II)alamin form was generated by reaction of the methylcobalamin form with homocysteine under aerobic conditions at 64°C. Assays were performed with 9.2 nM TM0268 in the presence of (●) equimolar TM0269 and 19 μM AdoMet; (□) AdoMet only; (△) TM0269 only; or (△) neither.

reconstituted with methylcobalamin (~110 turnovers/min). Thus far, we have not been able to generate TM0268 in the cob(II)alamin form without substantial irreversible loss of activity.

TM0269 is required for AdoMet-dependent remethylation of TM0268 in an electrochemical cell

Methylated TM0268 enzyme was 90% converted to the aquocob(III)alamin form by photolysis under aerobic conditions using a procedure derived for *E. coli* MetH (Jarrett et al. 1997). Protein was subsequently reduced in an electrochemical cell under anaerobic conditions at a potential of -0.450 V versus a standard hydrogen electrode in the presence of AdoMet and methyl viologen and in the presence or absence of TM0269. As judged from the absorbance properties of the protein solutions following gel filtration to remove excess AdoMet and methyl viologen, TM0268 was almost fully converted to the methylcobalamin form when equimolar TM0269 was present (Fig. 4, red line). If TM0269 was absent, the enzyme was isolated in the cob(II)alamin form after incubation in the electrochemical cell (Fig. 4, black line).

The degree of methylation was estimated by denaturation of the protein with guanidine HCl in the presence of cyanide, which releases cobalamin from the protein and converts aquocobalamin and cob(II)alamin, but not methylcobalamin, to dicyanocobalamin, which displays a characteristic visible spectrum (data not shown). By this test, >95% of the enzyme was remethylated in the presence of TM0269.

Discussion

The experiments reported here have documented functional similarities in the reactivation systems of the disparate *E. coli* and *T. maritima* MetH species, and the availability of the structures of both modules allows a detailed comparison. Although the structure of TM0269, deposited in the Protein Data Bank (1J6R), did not contain bound AdoMet, our functional studies and the conservation of the AdoMet-binding region in both structures allows identification of the AdoMet-binding site in TM0269.

Although the reactivation module of the *E. coli* enzyme reacts *in cis*, we have shown that the corresponding module of the *T. maritima* enzyme is actually a separate protein, TM0269, that reacts *in trans*. It is intriguing that the two-protein reactivation system from *T. maritima* functions adequately *in trans*, whereas it has not been possible to reactivate *E. coli* MetH when the activation domain has been cleaved from the catalytic domains by specific tryptic digestion (Drummond et al. 1993). Preliminary experiments suggest that the TM0268 and TM0269 proteins do not form tight aggregates; the proteins

Figure 4. Electrochemical methylation of TM0268 in the cob(II)alamin form requires TM0269. TM0268 in the cob(III)alamin form was generated by aerobic photolysis (Jarrett et al. 1996) and then incubated under argon in the presence of 1 mM AdoMet and 500 μM methylviologen at -0.450 V versus standard hydrogen electrode for 45 min at 25°C in the presence or absence of TM0269. Following incubation, the proteins were subjected to gel filtration to remove AdoMet and methylviologen, and the spectra were recorded. (Blue line) The absorbance of the hydroxycob(III)alamin form of TM0268 before electrochemical methylation; (black line) the TM0268 absorbance in the cob(II)alamin form after incubation in the absence of TM0269; (red line) the absorbance observed after incubation in the presence of equimolar TM0269. The black line indicates that incubation in the absence of TM0269 results in the formation of cob(II)alamin, which is characterized by an absorbance peak at 477 nm. The red line indicates that TM0268 is converted to methylcobalamin in the presence of TM0269 and AdoMet.

are readily separated from one another on a MonoQ anion exchange column or on sizing columns.

In attempts to induce incorporation of the cobalamin cofactor during expression of TM0268 in *E. coli* hosts, we co-transfected cells with plasmids bearing coding sequences for both TM0268 and TM0269. This tactic failed to increase significantly the yield of soluble TM0268 or the incorporation of cobalamin, implying that TM0269 does not exert any chaperone-like effects on TM0268. For human MetH, Yamada and coworkers (Yamada et al. 2006) have recently shown that cofactor incorporation can be enhanced significantly by coexpression with methionine synthase reductase, a large protein bearing a flavodoxin-like domain that serves as electron donor for reactivation of inactive cob(II)alamin MetH. The physiological reductase for Tm MetH might play a similar role, but, as already noted, it remains to be identified.

Structural similarities and differences between TM0269 and the reactivation domain of MetH from *E. coli* have been analyzed by comparing the structural models (Fig. 5). The lengths and sequences of the two functional modules are very different. The Tm protein is only 197 residues in length, compared with the 327 residues that comprise the reactivation domain of *E. coli* MetH. Sequence identities between residues aligned on the basis of their positions in the structures are also surprisingly low, being only ~15% identical. However, conserved conformations in central regions of the structure can account for the similar functional properties of the two proteins. A highly conserved core region includes the fourth strand of the major central sheet ($\beta 8$ in the *E. coli* sequence) (Fig. 5A) and the very long $\alpha 6$ -helix that carries some of the AdoMet binding determinants, as defined for the *E. coli* domain (Dixon et al. 1996). Also closely reproduced in both structures is a proline-rich “hook,” 1134–1140 in the *E. coli* sequence, and a hairpin formed by residues 1163–1173 of *E. coli* MetH and 175–186 of TM0269. The hook forms a highly conserved part of the binding site for the adenine moiety of AdoMet. Sequences of the hairpin are not well conserved, but the hairpin conformations are remarkably similar despite the insertion of one residue in the *T. maritima* structure. In the *E. coli* reactivation complex (Bandarian et al. 2002), this hairpin contacts the cobalamin-binding domain and pries the corrin ring away from His759. Dissociation of the His ligand from the cobalt of cobalamin presumably facilitates reduction of cob(II)alamin. Structural similarities in the hairpin of TM0269 imply that association of the cobalamin domain of TM0268 with TM0269 likewise perturbs cobalt ligation in TM0268.

In contrast, the degree of conservation between TM0268 and the first three modules of the *E. coli* MetH is significantly higher. A structure-based alignment of TM0268 and the first three modules of the human and

E. coli synthases was published as Supplemental Figure 1 in the paper reporting the structure of the substrate-binding domains of TM0268 (Evans et al. 2004). Of the 686 residues of TM0268, 149, or 22%, are identical in the human and *E. coli* amino acid sequences.

Structural comparisons also indicate differences in the AdoMet-binding determinants used by TM0269 and the reactivation domain of *E. coli* MetH. It is evident (Fig. 5A,C) that apart from the long helix, the polypeptide backbones are arranged differently in the vicinity of the Met moiety. No residue in TM0268 is equivalent to Asp946, which binds the amino group of AdoMet. Arg131, from a different segment of the structure, occupies a position that corresponds approximately to Asp946. Glu124 “replaces” the Arg1094 that binds the carboxyl group of AdoMet in the *E. coli* complex. These substitutions may, therefore, alter the orientation and conformation of bound AdoMet in its complex with TM0269, but possible conformations are restricted by the requirement to juxtapose the methyl group of AdoMet in an orientation suitable for transfer to cobalamin.

Long stretches of *E. coli* sequence are absent from TM0269 (Fig. 5C), primarily in the region shown at the bottom of the molecule in Figure 5A. In this region, the first 28 and last 43 residues of the *E. coli* sequence are missing, and the loops and strands comprising residues 1000–1050 in *E. coli* are contracted to 25 residues. Remaining deletions shorten the distal helices and remove some short helices (as shown in the upper left of Fig. 5A, $\alpha 7$ beginning at residue 1125 in the *E. coli* sequence and the 3_{10} -helix between $\alpha 3$ and $\alpha 4$ beginning at residue 962). Decreasing the length of surface loops is one strategy used by thermophiles to enhance protein stability (Thompson and Eisenberg 1999). The major changes (at the bottom of the view in Fig. 5A) occur where the cobalamin-binding domain is attached in *E. coli* MetH, suggesting that these features may be important for orienting the (covalently linked) modules of *E. coli* MetH with respect to one another. We speculate that they contribute to domain interfaces in some members of the ensemble of conformations required for catalysis (Bandarian et al. 2003; Evans et al. 2004).

Materials and Methods

Vectors for TM0268 and TM0269

A pBad vector specifying TM0269 with a MGSDKIHSHHHH leader sequence was constructed at the Joint Center for Structural Genomics. The coding region corresponding to the full-length TM0268 protein (residues 1–768) was PCR-amplified from genomic DNA (a gift of James Bardwell, University of Michigan, Ann Arbor). DNA sequence analysis verified that the PCR product coded for the correct sequence. The PCR product was inserted into Pet29a to generate a C-terminal His6 fusion with an AALV insertion.

Figure 5. (A) The structure of TM0269 (1J6R, yellow) is superimposed on the structure of the C-terminal domain of MetH from *E. coli* (1MSK, blue) (Dixon et al. 1996), with bound AdoMet displayed in ball-and-stick mode (1MSK). Residues used to match the structures are from a structurally conserved core that includes the *E. coli* helices $\alpha 5$ and $\alpha 6$ and strands $\beta 2$, $\beta 5$, and $\beta 8$ shown in the sequence alignment C. The hairpin that positions cobalamin in the structure of the reactivation complex from *E. coli* (1163–1173) is *above* and to the *right* of the adenine ring in this view. (B) Close-up view of the binding site for AdoMet in TM0269. (C) Structure-based sequence alignment of TM0269 and the activation domain of *E. coli* MetH. Similar structural elements that overlay each other or show small displacements in A are blue, and identities are highlighted. Italicized sections of the *T. maritima* sequence are structurally dissimilar to the *E. coli* structure and demonstrate the shortened loops of the thermophile.

Expression and purification of TM0268

Protein was overexpressed in transformed C41 cells grown in LB medium at 37°C. Expression levels were higher in this host strain than in BL21 (DE3) Gold cells, and the protein eluted from Zn-NTA columns was purer. At 0.6–0.7 OD₆₀₀, cultures were induced with 750 μ M IPTG and allowed to grow for 8 h at 37°C. Cells were lysed by sonication. All buffers used for purification and dialysis contained 1 mM TCEP. The lysate was heated for 10 min at 70°C and then centrifuged at 96,000g for 75 min in a Beckman L-70 ultracentrifuge. The supernatant was passed through a 0.45- μ m filter and loaded onto a Hi-Trap Zn-NTA column (Amersham) in 100 mM Tris buffer (pH 8.0) containing 500 mM NaCl. After washing the column with loading buffer, the tagged protein was eluted with 1.5 M glycine and dialyzed against 50 mM Tris (pH 8.0). The protein was concentrated with a 30K cutoff Amicon concentrator to 60 mg/mL (as determined by the DTNB assay) and was 100% zinc replete according to the zinc assay. Cobalamin content after reconstitution was determined from the visible spectrum (Fig. 1), as described below.

Expression of TM0269 and coexpression of both proteins

To optimize expression of soluble protein and to examine effects of coexpression on yields, the pBad and pET 29a vectors carrying TM0269 and TM0268 were transfected and cotransfected into several cell lines, including BL21 (DE3), BL21 Gold (DE3), and C41 (DE3), and Rosetta. Routinely, protein expression was induced with 750 μ M IPTG (for TM0268) and/or 0.02% arabinose (for TM0269) in the BL21 (DE3) Gold cells.

Analyses of protein concentration, zinc content, and cobalamin content

Protein concentration was determined by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 6 M guanidinium hydrochloride buffered with 0.05 M Tris chloride (pH 8.0) as described (Bandarian and Matthews 2001). This procedure measures the three cysteines in TM0268 and has provided accurate estimates of MetH concentrations in our hands. The extinction coefficient at 412 nm for the thio-2-nitrobenzoate anion released on reaction with cysteine thiols was taken to be 13,600 M⁻¹·cm⁻¹. It was necessary to modify the published procedure (Bandarian and Matthews 2001) to correct for the absorbance of cobalamin at 412 nm. Zinc content was determined by titrating with 4-(2-pyridylazo)resorcinol in the presence of 4-hydroxymercuribenzoate (Aldrich) (Goulding and Matthews 1997) except that this compound was substituted for 4-hydroxymercuriphenylsulfonate, which is no longer available. Concentrations of bound methylcobalamin were determined from absorbance at 536 nm using an extinction coefficient of 8910 M⁻¹·cm⁻¹.

Reconstitution of holo-TM0268

In a typical reconstitution of apo-TM0268, a 200- μ L aliquot of 0.3–0.5 mM apo-protein in a 1-mL microfuge tube was preheated for 2 min at 60°C in 50 mM Tris chloride buffer (pH 8). Following addition of a 10-fold excess of methylcobalamin, the sample was maintained for an additional 3 min at 60°C. Excess methylcobalamin was removed by eluting the protein from a Econo-pac 10DG Bio-gel P-6 column (Bio-Rad) equilibrated with 10 mM potassium phosphate buffer (pH 7.2). The protein was concentrated in the same buffer and stored at –80°C.

Conversion of methylated enzyme to the cob(II)alamin form

Reaction with Hcy to form methionine demethylates the methylcobalamin enzyme. Under aerobic conditions, the resulting cob(I)alamin enzyme is converted to the cob(II)alamin species. Conversion from methylcobalamin to the cob(II)alamin species can be observed spectrophotometrically by changes in absorbance at 536 nm (the absorbance maximum for the methylcobalamin of TM0268) or 477 nm [the absorbance maximum for the cob(II)alamin form].

Assays of methionine synthase activity

Activity was measured at selected time points by spectrophotometric determination of the methenyl-tetrahydrofolate derived from the product H₄folate by reaction with formic acid (Jarrett et al. 1997). Assay mixtures contained 100 mM potassium phosphate buffer (pH 7.2), 25 mM dithiothreitol, 0.019 mM AdoMet (where desired), 0.05 mM hydroxocobalamin, 0.5 mM Hcy, and 0.25 mM CH₃-H₄folate.

Two different assay protocols were used in these studies: (1) To determine temperature dependence of activity, mixtures containing the enzyme(s) and all components except the CH₃-H₄folate substrate were preincubated under aerobic conditions for 2 min at the desired temperature, and reactions were initiated by addition of CH₃-H₄folate, which was added last because of its lability at elevated temperatures (Indrawati et al. 2005). Use of this protocol allows deactivation (by reaction with Hcy) and reactivation (in the presence of AdoMet, TM0269, and the chemical reducing system) during temperature equilibration. (2) To follow rates of reaction and the dependence of rates on AdoMet and/or TM0269 at 64°C, all components of the assay except the protein(s) were combined, flushed with argon for 10 min, and brought to the desired assay temperature by incubation for 2 min. Enzyme(s) were added to initiate turnover.

Electrochemical methylation

TM0268 in the hydroxycob(III)alamin form was converted to the methylcobalamin form by reductive methylation in the presence of AdoMet, as previously described (Jarrett et al. 1996). The enzyme (50–200 μ M in 0.1 M potassium phosphate buffer at pH 7.2, containing 0.2 M KCl) was mixed with AdoMet (1 mM) and methylviologen (500 μ M) under argon in an electrochemical cell and poised at –450 mV versus the standard hydrogen electrode for 45 min at 25°C. After reaction, the protein was purified in the dark by gel filtration and then equilibrated with 10 mM potassium phosphate buffer (pH 7.2) and concentrated in a Centricon 30 Concentrator (Amicon).

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