Assignment of Enzymatic Function to Specific Protein Regions of Cobalamin-Dependent Methionine Synthase from *Escherichia coli*†

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ABSTRACT: Cobalamin-dependent methionine synthase catalyzes methyl group transfer from methyltetrahydrofolate to homocysteine to form tetrahydrofolate and methionine, and the cobalamin prosthetic group serves as an intermediate methyl carrier. Enzyme possessing cobalamin in the cobalt(II) oxidation state is inactive, and this form is activated by one-electron reduction coupled to methylation by *S*-adenosylmethionine (AdoMet). The enzyme from *Escherichia coli* has been divided into separable fragments by limited proteolysis with trypsin, and the contribution of each of these fragments to substrate binding and catalysis has been evaluated. The 37.7-kDa carboxyl-terminal domain binds AdoMet, and this was demonstrated through covalent modification with radiolabeled AdoMet during ultraviolet irradiation. Following reductive activation with AdoMet, the enzyme was digested with trypsin and a 98.4-kDa amino-terminal fragment was isolated. It retained at least 70% of the activity of the intact enzyme and must therefore possess determinants sufficient for the binding of methyltetrahydrofolate and homocysteine, as well as residues required for catalysis. However, when the cobalamin was oxidized to the cob(II)alamin state, the 98.4-kDa fragment could not be reductively remethylated with AdoMet. A purified, 28-kDa domain within the 98.4-kDa fragment retained bound cobalamin and therefore must play a central role in catalysis, but the isolated 28-kDa domain retained no catalytic activity. Because AdoMet binds to a different domain of the protein than methyltetrahydrofolate and homocysteine, the enzyme probably uses conformational flexibility to allow the cobalamin access to the required methyl donor or acceptor at the appropriate time in catalysis. These results provide an explanation for the exclusion of AdoMet from the normal turnover cycle and suggest how AdoMet, methyltetrahydrofolate, and homocysteine may be bound simultaneously.

Cobalamin-dependent methionine synthase from *Escherichia coli* is an unusually large, monomeric enzyme. With a mass of 136.1 kDa (Drummond et al., 1993), it is over 3 times the size of the average *E. coli* protein (Neidhardt, 1987). Its gene has been cloned, sequenced, and overexpressed (Banerjee et al., 1989; Old et al., 1990), and recently the DNA sequence has been corrected to provide a predicted amino acid sequence consistent with the isolated protein product at the carboxyl terminus (Drummond et al., 1993). Although considerable progress has been made toward understanding the chemical mechanism of the enzyme (Taylor & Weissbach, 1973; Banerjee & Matthews, 1990), relatively little is known about how this large monomer is assembled into an enzyme capable of carrying out the complex set of reactions it catalyzes. The enzyme uses a noncovalently bound cobalamin prosthetic group to catalyze a methyl group transfer from 5-methyltetrahydrofolate (CH_3-H_4folate)[1] to homocysteine, and this is achieved indirectly as the sum of the two half-reactions given in eqs 1 and 2.

$$\text{E·Cob(I)alamin} + CH_3\text{-}H_4\text{folate} \rightarrow \text{E·Methylcobalamin} + H_4\text{folate} \quad (1)$$

$$\text{E·Methylcobalamin} + \text{Homocysteine} \rightarrow \text{Methionine} + \text{E·Cob(I)alamin} \quad (2)$$

The highly reactive, enzyme-bound cob(I)alamin nucleophile (Taylor & Hanna, 1970; Fujii & Huennekens, 1979) removes the methyl group from CH_3-H_4folate (eq 1), and cob(I)alamin then acts as the leaving group as homocysteine accepts the methyl group to form methionine (eq 2).

By this formal mechanism, methionine synthase generates two metabolically essential products. The tetrahydrofolate thus formed may be charged with one-carbon units of other oxidation states to generate methylenetetrahydrofolate or formyltetrahydrofolate, and these cofactors serve as biosynthetic precursors, most notably in nucleotide biosynthesis. In *E. coli*, the product methionine may be consumed in protein biosynthesis or converted to *S*-adenosylmethionine (AdoMet), a highly versatile and potent methyl donor. Because the cobalamin-dependent methionine synthase from mammals uses the same prosthetic group, catalyzes the same reaction, and displays the same catalytic requirement for AdoMet and a reducing system (Loughlin et al., 1964; Taylor & Weissbach, 1973), the enzyme from *E. coli* may also provide a reasonable model for study of the structure and function of the mammalian counterpart.

Methionine synthase requires two different methyl donors, and each has a specific role in catalysis (Figure 1). AdoMet appears to act as a thermodynamic activator, since the role

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid, sodium salt; FPLC, fast protein liquid chromatography; Hcy, L-homocysteine; CH_3-H_4folate, (6-*R*,*S*)-5-methyltetrahydrofolate monoglutamate; TLCK, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone hydrochloride; Tris, tris(hydroxymethyl)aminomethane.

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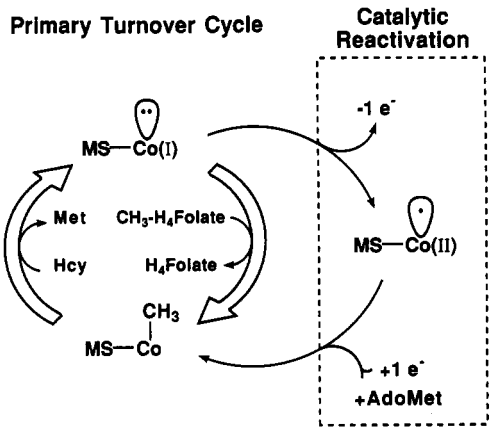

FIGURE 1: Schematic summary of the chemistry carried out by methionine synthase (MS). In normal catalysis, the prosthetic group cycles between cob(I)alamin and methylcobalamin (abbreviated Co-(I) and Co-CH_3, respectively). Homocysteine demethylates methylcobalamin to generate methionine and cob(I)alamin, and this highly reactive intermediate is rapidly remethylated by CH_3-H_4folate. The broad arrows reinforce the observation that turnover is largely described by these events. However, the enzyme-bound cob(I)alamin may also become oxidized to cob(II)alamin, and this enzyme form is catalytically inactive. The enzyme is reactivated by reductive methylation involving AdoMet and one electron. The dashed box is used to emphasize the fact that methyl group donation from AdoMet to the cobalamin is physically segregated from catalysis (see text), but that both substrates and AdoMet must have access to the central cobalt.

of this cofactor is to convert the catalytically inactive cob-(II)alamin to methylcobalamin. The highly unfavorable reduction of cob(II)alamin to cob(I)alamin ($E^{\circ\prime} = -526$ mV vs the standard hydrogen electrode) can be coupled to the highly favorable methylation by the methylsulfonium cation of AdoMet (Banerjee et al., 1990a). However, because AdoMet is generated from methionine and ATP (Cantoni & Durell, 1957), remethylation of homocysteine with this agent would be energetically very costly and biosynthetically futile. Instead, the primary methyl donor during turnover is 5-methyltetrahydrofolate. Demethylation of methylcobalamin with homocysteine yields the highly reactive cob(I)alamin, and this intermediate is rapidly remethylated by CH_3-H_4folate (Banerjee et al., 1990b). This latter process accounts for the bulk of remethylation, but when catalysis is intercepted by an oxidant and enzyme-bound cob(I)alamin is converted to cob-(II)alamin, reactivation by remethylation with AdoMet is required. This is clearly a minimal mechanism and does not rule out additional roles for AdoMet, either catalytic or regulatory.

We have now assembled an "ultralow-resolution" structure for methionine synthase by assigning functions to isolable domains generated by limited proteolytic digestion. A "domain" is defined here as a proteolytically stable, isolable fragment of the enzyme that retains some specific function required for overall enzyme competence. These functions include substrate and prosthetic group binding as well as catalytic activity. We posit that the extensive primary structure folds into a set of domains that each contribute specific binding and catalytic properties, and that the enzyme physically segregates the two methyl pools upon which it draws for catalysis. The catalytic activator AdoMet can thus be excluded from normal turnover, but may be bound in reserve on a 37.7-kDa carboxyl-terminal domain and called upon when normal turnover is interrupted by oxidation of the cobalt. Once activated, turnover is effected by an amino-terminal 98.4-kDa fragment that includes the cobalamin-binding domain.

EXPERIMENTAL PROCEDURES

Materials. [*Methyl*-^{14}C]CH_3-H_4folate (57.5 mCi/mmol), provided as the barium salt, and [*methyl*-^{3}H]AdoMet (80 Ci/mmol), provided as the chloride salt, were purchased from Amersham. Radiolabeled CH_3-H_4folate was diluted to 1200 dpm/nmol with unlabeled CH_3-H_4folate, purchased as the calcium salt from Sapec Chemical Co., Lugano, Switzerland. Dithiothreitol was obtained from BioRad, while trypsin, trypsin inhibitor, and other chemical reagents were purchased from Sigma.

Purification of Methionine Synthase and Its Domains. Recombinant methionine synthase (MetH) from *E. coli* strain DH5αF′/p4B6.3 was isolated as previously reported (Banerjee et al., 1989). Details of the chromatographic purification of the individual domains are presented in Drummond et al. (1993).

Conversion of Enzyme-Bound Methylcobalamin to Cob(II)-alamin. Methylated methionine synthase (7 nmol, 10 μM) in 700 μL of aerobic 100 mM potassium phosphate buffer (pH 7.2) with 20 mM dithiothreitol and 1 mM EDTA was treated with L-homocysteine (1.4 μmol, 2 mM) to give a spectral change consistent with the formation of enzyme-bound aquocob(III)alamin. The solution was immediately made anaerobic by exchanging the atmosphere with argon gas by repeated cycles of evacuation followed by argon replacement at 4 °C. Reduction to cob(II)alamin was monitored spectrally by the increase in absorbance at 480 nm over the course of 2 h at ambient temperature. The free thiols were removed by 3 cycles of ultrafiltration to ~100 μL in a Centricon 30 microconcentrator (Amicon) followed by dilution with 2.0 mL of 50 mM phosphate buffer containing 1 mM EDTA at 4 °C.

Digestion of Enzyme for Direct Assay of Fragments. Aliquots of 100-μL volume containing methionine synthase (2 μM, 27.4 μg) and trypsin (0.14 μg, 0.5% w/w) in potassium phosphate buffer, pH 7.2, were incubated for 30 min at ambient temperature, and portions were added directly to the assay solution as described below. For electrophoretic analysis, the digestion was terminated by the addition of TLCK (1 μL of a 1 mg/mL stock in water). Because high levels of TLCK inhibited enzyme activity, this reagent was avoided in all experiments where turnover was monitored.

Photo-cross-linking of Methionine Synthase with [Methyl-^{3}H]AdoMet. Irradiations were carried out in flat-bottomed 96-well cell culture plates (Costar, Cambridge, MA) on a bed of ice. Typically, a well contained methionine synthase (4 μg, 1.25 μM) and [*methyl*-^{3}H]AdoMet (2.5 μM, 80 Ci/mmol) in 25 μL of 50 mM potassium phosphate buffer at pH 7.2. After a 15-min incubation at ambient temperature, the plate was placed on ice and a small ultraviolet lamp (UVP Corp., Model UVGL-58, San Gabriel, CA) was secured directly above the wells as close as possible to the protein solution (~1 cm). The solution was irradiated for 20 min at 254 nm (surface output, 300 μW/cm^2). The enzyme or fragment mixtures were then separated on denaturing 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. After being stained with Coomassie blue dye, the gels were soaked for 1.5 h in En^{3}Hance (New England Nuclear), allowed to stand for 20 min in distilled water, dried, and exposed to X-OMAT AR film (Kodak) for 1–3 days.

Assay of Methionine Synthase and Its Peptide Fragments. The assay method is a modification of the procedure of Weissbach et al. (1963). The following reagents were added to Pyrex test tubes (1.5 × 10 cm) to achieve a final volume of 3.2 mL and the given final concentrations: potassium

phosphate buffer, pH 7.2, 100 mM; dithiothreitol, 25 mM; L-homocysteine, 1 mM; AdoMet, 19 μM; [*methyl*-^{14}C]CH_3-H_4folate (1200 dpm/nmol), 250 μM; aquocobalamin, 50 μM; and methionine synthase or the 98.4-kDa fragment thereof, 4.4 nM (methylcobalamin) to 5.9 nM (cob(II)alamin). The water, buffer, dithiothreitol, homocysteine, and AdoMet (where specified by the individual figure legends) were combined, and the tube containing the mixture was fitted with a rubber septum. Argon was gently bubbled through the solution for 5 min to remove most of the oxygen before the addition of [*methyl*-^{14}C]CH_3-H_4folate and aquocobalamin. The tube was incubated at 37 °C for 5 min before turnover was initiated, either with methionine synthase or a domain derived from the enzyme. Aliquots of 500 μL were withdrawn at the given intervals and quenched upon addition to a test tube maintained at 90 °C in a heating block. After 3 min the tubes were cooled on ice, and the contents were diluted with 500 μL of water and passed over an AG1-X8 anion-exchange column in a Pasteur pipette to separate [*methyl*-^{14}C]methionine from unreacted [*methyl*-^{14}C]CH_3-H_4folate. The columns were washed twice with 1.0 mL of water, and methionine in the eluate was quantitated by scintillation spectrometry.

Characterization of Domains. The localization of domains in the primary amino acid sequence is largely the result of other work. Drummond et al. (1993) redefined the carboxyl-terminal 37.7 kDa by a combination of electrospray mass spectrometry and Edman degradation, and this domain includes residues 897–1227. The 98.4-kDa domain has been shown to end with arginine 896, and the mass given was deduced from the DNA sequence of Old et al. (1990). This is consistent with the apparent molecular weight determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and assumes that the 98.4-kDa fragment possesses the same amino terminus as the intact protein. The tryptically generated cobalamin-binding domain begins with threonine 643 (Banerjee et al., 1989) and also ends with arginine 896. This domain accounts for the 28.0-kDa mass, predicted from the DNA sequences, that runs from the N-terminus of the cobalamin-binding domain to the end of the 98.4-kDa domain.

RESULTS

Localization of AdoMet Binding. To determine the location of AdoMet binding in methionine synthase, we used a procedure for covalent linkage of AdoMet to proteins first described by Yu (1983) for labeling phenylethanolamine *N*-methyltransferase. This method involves ultraviolet irradiation of a noncovalent complex of radiolabeled AdoMet and the protein to be labeled. More recently, a series of methyltransferases that utilize AdoMet have been covalently modified by ultraviolet irradiation in the presence of radiolabeled AdoMet. These include *Eco*RII methyltransferase (Som & Friedman, 1991), CheR (chemoreceptor) methyltransferase (Subbaramaiah & Simms, 1992), and guanidinoacetate methyltransferase (Takata & Fujioka, 1992), and in these examples the position of the radiolabel has been localized in the primary sequence. We have found that methionine synthase is specifically labeled with [*methyl*-^{3}H]-AdoMet during a 20-min exposure to short-wavelength ultraviolet light (Figure 2). When methionine synthase was first cut with trypsin into two fragments, the carboxyl-terminal 37.7-kDa domain was also labeled with an efficiency similar to the intact protein, both in the digested mixture and following purification away from the larger fragment. These results

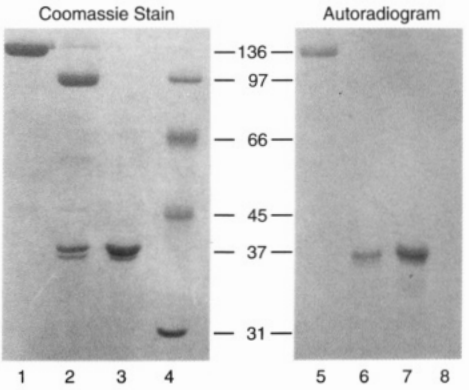

FIGURE 2: Cross-linking of [*methyl*-^{3}H]AdoMet to methionine synthase and to the carboxyl-terminal 37-kDa domain. Lanes 1–4: methionine synthase holoprotein and tryptic fragments and molecular weight standards stained with Coomassie blue dye following a 20-min ultraviolet irradiation at 4 °C in the presence of [*methyl*-^{3}H]-AdoMet. The standards were irradiated in the presence of labeled AdoMet in an identical manner. Lanes 5–8 show a fluorogram of this same gel. Lanes 1 and 5, methionine synthase holoprotein; lanes 2 and 6, a mixture of amino-terminal 98.4- and 37.7-kDa domains formed by tryptic digestion. Further tryptic cleavage also removes four amino acid residues from the amino terminus of the 37.7-kDa domain to yield a 37.2-kDa domain (Drummond et al., 1993); lanes 3 and 7, purified C-terminal 37-kDa domains; lanes 4 and 8, protein standards with the indicated mass values in kDa. None of the proteins label in the absence of UV irradiation (data not shown), and labeling is not inhibited by high salt (100 mM KCl) or dithiothreitol (25 mM).

suggest that all of the determinants required for AdoMet binding and cross-linking are present in the 37.7-kDa domain. The specificity of the cross-linking reaction is evident from the lack of cross-linking either to the 98-kDa domain or to the proteins used as molecular weight standards.

For methionine synthase, the specific residue(s) involved have not been characterized, although labeling is presumed to involve a methyl transfer from [*methyl*-^{3}H]AdoMet to the enzyme. When [^{35}S]AdoMet of the same specific radioactivity was synthesized by the method of Sumner et al. (1986) and the cross-linking repeated, labeling by [^{35}S]AdoMet was barely detectable in comparison with labeling by tritiated AdoMet (Christopher Harris, unpublished observation). This is consistent with the observation that methyl group transfer is the mode of labeling for the *Eco*RII and CheR methyltransferases cited above, although the mechanism of this process is not well understood. The efficiency of labeling methionine synthase with [*methyl*-^{3}H]AdoMet in these experiments is low (~0.1%), and this has hindered ongoing efforts to identify the specific site of labeling. Labeling with low efficiency is sufficient for the primary purpose here, i.e., the initial localization of AdoMet binding to a specific fragment of the enzyme.

Localization of Catalytic Determinants. According to the model in which AdoMet is a thermodynamically activating methyl donor for methionine synthase, repeated turnovers with CH_3-H_4folate as the methyl donor should occur following a single reductive methylation by AdoMet (Taylor & Weissbach, 1973). Methionine synthase was therefore reductively methylated with AdoMet in an electrochemical cell as previously described (Luschinsky et al., 1992). The excess free AdoMet was removed by repeated ultrafiltration of the enzyme solution to a small volume followed by 10-fold dilution into buffer free of AdoMet. This enzyme stock was then used in a series of assay experiments designed to assign catalytic activity to the various tryptic fragments. Figure 3 shows the activity of the holoenzyme when added to an anaerobic assay solution. In

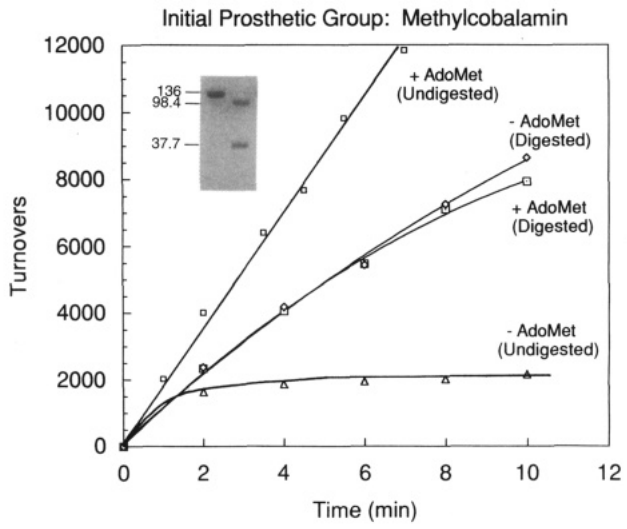

FIGURE 3: Dependence of activity on AdoMet and on the integrity of the primary structure. Turnovers express the number of [*methyl*-^{14}C]methionine molecules formed from [*methyl*-^{14}C]CH_3-H_4folate per molecule of methionine synthase. In the absence of AdoMet, the methylated enzyme begins to turn over normally, but rapidly accumulates as the inactive cob(II)alamin. The holoprotein plus AdoMet line is characteristic of fully active enzyme; here, the role of AdoMet is evident as oxidized cobalamin is reactivated in the assay system. When the enzyme is digested with 0.1% (w/w) trypsin (see inset), two domains are generated and the mixture retains catalytic activity that is independent of AdoMet.

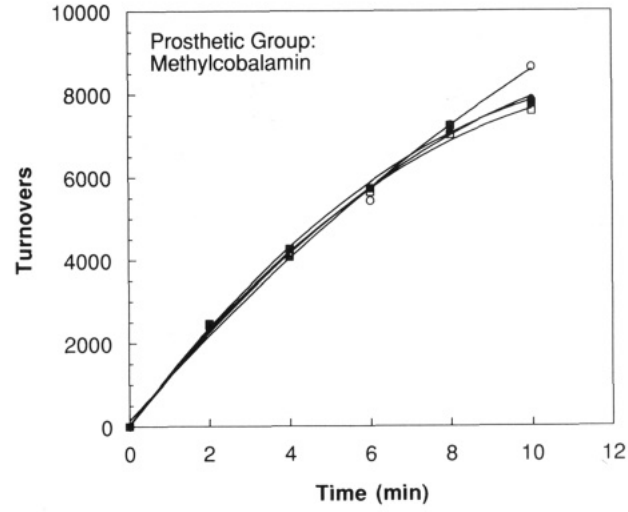

FIGURE 4: Contribution of the 37.7-kDa domain to normal turnover. Methylated holoprotein was digested with trypsin, and the 98.4-kDa domain was purified by anion-exchange chromatography. A digested mixture of domains was assayed in the presence (○) and absence (●) of AdoMet. The purified 98.4-kDa domain was then assayed in the presence (□) and absence (■) of AdoMet. Identical amounts of protein were assayed, on the basis of the spectrum of the purified domain and assuming that the extinction coefficient for vitamin B_{12} bound to the 98.4-kDa domain was unchanged from that of the holoprotein. The activity observed for the 98.4-kDa domain is independent of the C-terminal domain, suggesting that the AdoMet-binding domain does not contribute to the primary catalytic cycle.

the presence of substrates, Adomet, and a reducing system (dithiothreitol serves as a reducing agent, and electron transfer to the enzyme is thought to be mediated by free, reduced cobalamin), the enzyme rapidly reaches steady state. However, in the absence of AdoMet, the production of methionine ceases over the course of several minutes. The implication is that the cob(I)alamin is becoming oxidized to cob(II)alamin during the normal course of turnover, and in the absence of AdoMet the enzyme cannot be reductively activated back to the turnover-competent methylcobalamin form.

When the enzyme was cleaved after arginine 896 by trypsin and assayed as a mixture of two fragments at the same concentration as the intact protein, three important observations were made. First, the mixture retained at least 70% of the initial activity of the intact protein (Figure 3), on the basis of the relative number of turnovers that occurred over the first 2 min. Second, the activity of the tryptic mixture became *independent* of AdoMet, and the formation of methionine was not linear over time, suggesting that the domain was slowly converted to a turnover-incompetent form that could not be reactivated by AdoMet. Third, in the absence of AdoMet, the turnover of the cleaved protein was longer lived than the turnover of the intact protein. This suggests that the carboxyl-terminal 37.7-kDa domain influences the oxidation that leads to inactivation, either by directly accepting electrons from the 98.4-kDa domain or by simply allowing greater access of exogenous oxidants to the cobalamin. We conclude that a single break in the primary sequence does not destroy normal enzyme activity. We also infer that the intact peptide backbone is required for the catalytic activation by AdoMet, but caution is in order for this conclusion; the tryptic cut does introduce two new charged groups as the protein is hydrolyzed, and this change alone could account for loss of the ability to reactivate with AdoMet.

Because the domains were readily separable by anion-exchange chromatography, we also assessed the activity of the purified, methylated 98.4-kDa domain (Figure 4). Identical amounts of protein were assayed, on the basis of the spectrum of the purified components and assuming that the extinction coefficient for methylcobalamin bound to the 98.4-kDa domain was the same as for the holoprotein. This value is in agreement with the amount of protein present, based on a Bradford protein assay, implying that the fragment retained full occupancy by the cobalamin. As shown in Figure 4, the purified 98.4-kDa domain retained all of the catalytic activity present in the digested mixture of domains. Again, this activity was independent of added AdoMet. We infer that the amino-terminal 98.4-kDa domain retains most, if not all, of the determinants needed for catalysis involving CH_3-H_4folate and homocysteine. The presence of the 37.7-kDa carboxyl-terminal domain, whether or not it remains associated with the 98.4-kDa domain, does not affect this activity. This observation is consistent with the role proposed for the 37.7-kDa domain, namely, the binding of AdoMet and reactivation of the enzyme by reductive methylation.

In order to further test this model, a complementary set of experiments was performed where the enzyme-bound vitamin B_{12} was first converted to the cob(II)alamin oxidation state. The enzyme activity is now dependent upon activation by AdoMet before any turnovers may occur (refer to Figure 1), because the cob(II)alamin state is catalytically incompetent. As shown in Figure 5, enzyme with bound cob(II)alamin added to an anaerobic assay solution rapidly reached steady state. The observed lag phase prior to reaching steady state reflects the reactivation step, presumed to be slow compared to turnover with CH_3-H_4folate and homocysteine. Now the observed turnover is indeed absolutely dependent upon added AdoMet. The 98.4-kDa domain with bound cob(II)alamin was inactive in the absence of AdoMet, in contrast to the domain containing methylated cobalamin. In the presence of AdoMet, the mixture of 98.4- and 37.7-kDa domains retained approximately 5% of the original activity. This is likely to have resulted from a small amount of undigested holoprotein remaining in the digest, rather than to an activity inherent to the domain

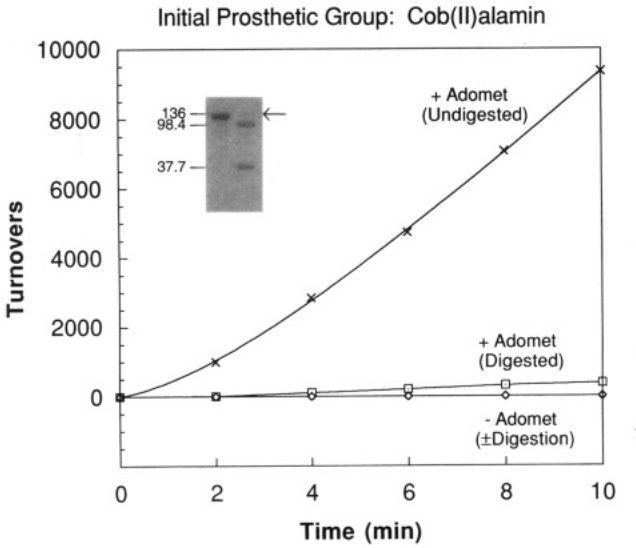

FIGURE 5: Direct demonstration of the mechanistic role of the carboxyl-terminal 37.4-kDa domain. Methionine synthase in the cob(II)alamin form was introduced into an anaerobic assay system. In the absence of AdoMet, no turnover was observed. In the presence of AdoMet, a brief lag phase was observed as the enzyme reached steady-state turnover. Tryptic digestion reduced the observed activity to 5% of the original value, and this is likely due to the residual, undigested holoprotein (see inset). The purified 98-kDa domain, prepared from holoenzyme in the cob(II)alamin form, was also inactive in the absence of AdoMet.

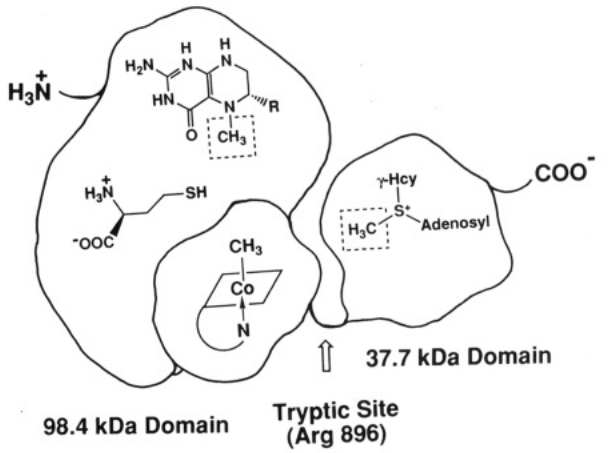

FIGURE 6: Proposed structure for methionine synthase. The trypsin acts on a relatively exposed site to divide the enzyme into two fragments of 98.4 and 37.7 kDa. The carboxyl-terminal domain independently binds and is chemically labeled by AdoMet. The amino-terminal 98.4-kDa domain contains all the structural features necessary for catalysis involving CH_3-H_4folate and homocysteine. This domain may be further proteolyzed to yield a stable 28-kDa domain that binds cobalamin but is catalytically incompetent. The two different methyl donors have been boxed to highlight the two independent methyl pools from which the enzyme draws to methylate the cobalamin. AdoMet is excluded from normal catalysis but must be able to access and remethylate cob(II)alamin bound to the enzyme. "R" in the figure represents the remainder of the *p*-aminobenzoate monoglutamate.

or to an interaction between domains in the crude digest. A small amount of undigested holoprotein is visible in the digest (see inset, Figure 5), and this reflects a slower rate of proteolysis for enzyme in the cob(II)alamin state. Further, when the 98.4-kDa domain from this experiment was purified, this residual activity was eliminated.

Inactivity of the Cobalamin-Binding Domain. Inherent to the holoprotein and the 98.4-kDa domain is the ability to bind the cobalamin prosthetic group. However, two overlapping fragments of ~28 kDa from the enzyme have been characterized that also contain the cobalamin. Luschinsky et al. (1992) have crystallized one domain, beginning with alanine 649, and are attempting to solve the structure by X-ray crystallography. Banerjee et al. (1989) reported the isolation of a closely overlapping domain generated by tryptic proteolysis, and the sequence boundaries of this domain have now been established (Drummond et al., 1993). We digested methylated holoprotein with trypsin to evaluate the kinetic competence of the tryptic fragment. Because the cobalamin-binding domain is both smaller and more basic than the holoprotein and other domains, it may be separated from them by either size-exclusion or anion-exchange chromatography. When the domain containing methylated cobalamin was assayed as described for the intact protein and the 98.4-kDa domain, no activity was observed even at a domain concentration 10-fold higher than that used for the 98.4-kDa domain. When homocysteine was added to the methylated 28-kDa domain and the cobalamin was monitored spectrally, no evidence of demethylation was evident over the course of 1 h (data not shown). In contrast, homocysteine rapidly demethylated the active 98.4-kDa domain and the holoprotein. This result suggests that the cobalamin-binding subdomain lacks key determinants for binding substrates or for catalytic function. One reasonable hypothesis is that many of these determinants are contributed by the amino-terminal 69-kDa polypeptide region that lies immediately upstream from the cobalamin-binding domain in the primary sequence.

DISCUSSION

The diagram provided in Figure 6 is a summary of our results, and it is rationalized as follows: limited tryptic proteolysis revealed a hypersensitive region between arginine 896 and lysine 897 that split the protein into two fragments. The carboxyl-terminal 37.7-kDa domain could be purified, and it was labeled covalently by ultraviolet irradiation in the presence of tritiated AdoMet with an efficiency similar to the labeling of intact protein. When the methylated holoprotein was cleaved by limited tryptic digestion, the 98.4-kDa domain retained the ability to catalyze methylation of homocysteine by CH_3-H_4folate, and this activity was independent of AdoMet. These observations are consistent with the assignment of AdoMet binding to the carboxyl-terminal domain and directly localize most (if not all) of the essential binding and catalytic determinants for methyl transfer from CH_3-H_4folate to homocysteine to the 98.4-kDa domain. When the enzyme was more completely digested with trypsin, a stable 28-kDa domain was isolated containing the cobalamin prosthetic group. This fragment, however, retained neither catalytic activity nor the ability to be demethylated by homocysteine. It is likely that some of the protein structure responsible for substrate binding and catalysis is contributed by the remaining amino-terminal 69-kDa polypeptide.

Even with this set of domain assignments, the means by which the protein structure brings together the turnover components with the cobalamin prosthetic group at the appropriate time to effect catalysis is not clear. Methionine synthase must provide a protein environment capable of binding the cobalamin and modulating the chemistry of the carbon–cobalt bond. However, the cobalamin must also be able to physically access each of the methyl donors and acceptors in a spatially precise manner, and the protein must be able to catalyze each of the different methyl-transfer reactions. Stereochemical analysis of the methyl transfer from CH_3-H_4folate to homocysteine showed retention of configuration in the chiral methyl group (Zydowsky et al., 1986),

and this supports a double-displacement mechanism involving successive S_N2 transfers between the substrates and the central cobalt. The enzyme must therefore orient the homocysteine sulfur along the axis of the cobalt to methyl bond, directly above the methyl group. Yet later in turnover, when the cob(I)alamin nucleophile becomes remethylated by CH_3-H_4folate, the same space relative to the cobalt must be occupied by the methyl group and the remainder of the folate. Both substrates therefore must occupy the same region in space during turnover.

Considering that the cobalamin contains a large, highly conjugated planar surface, a mechanism must exist to allow the upper axial face of this molecule to contact different protein regions at different times. Specifically, when in the cob(II)alamin state, the B_{12} must access the 37.7-kDa domain to become methylated, but during turnover this domain must be excluded. Cob(II)alamin does differ in formal charge from cob(I)alamin, and this may be a discriminating factor in the conformational state of the unmethylated enzyme. Cob(II)alamin also differs from methylcobalamin in that it lacks the methyl ligand, and therefore it is a potential acceptor of ligands donated by the protein from the carboxyl-terminal domain. These proposals lack experimental support, but they offer a means by which the cobalamin could recognize or exclude the AdoMet-binding domain as required.

It is interesting to reconsider the requirement for AdoMet in catalysis. While its contribution is readily rationalized on thermodynamic grounds, providing reentry into catalysis following oxidation, it is evident from this work that a considerably smaller enzyme (98.4 kDa) is capable of carrying out the primary catalysis of homocysteine remethylation. In an obligate anaerobe, the requirement for AdoMet may thus be relaxed, especially if one considers that the chances of cob(I)alamin interception by oxidation would be greatly diminished. Also, the ambient potential of the cell would be decreased (more reducing), and direct reduction of cob(II)alamin to cob(I)alamin would be more reasonable. We speculate that a domain capable of AdoMet binding and cobalamin methylation may be an adaptation to life in a semiaerobic cellular environment. By this model, cobalamin-dependent methionine synthase from organisms that are obligate anaerobes may well resemble the 98.4-kDa fragment from the *E. coli* enzyme, and the activity of these enzymes would be predicted to be independent of AdoMet. Unfortunately, cobalamin-dependent methionine synthase has not yet been purified from a prokaryote that is an obligate anaerobe, and this prediction remains to be tested.

In the *E. coli* enzyme, the catalytic determinants for both turnover and reductive activation are contained within a long primary sequence. In this work, we propose that the determinants are physically segregated by localizing the protein structure required for turnover on a separate fragment from that required for reductive activation with AdoMet. Alternatively, the reductive activation requirement involving AdoMet could be provided by a second subunit in a heterodimeric structure. Since neither the molecular mass nor the structural makeup of the mammalian cobalamin-dependent enzymes has been well characterized, this class of enzymes could also provide an example of a different structural solution to a formally identical catalytic cycle. This model is at least consistent with the observation that methionine synthase from human placental tissue is a heterodimeric structure (Utley et al., 1985), proposed to consist of subunits with masses of 90, 45, and 35 kDa that form a native structure of 160 kDa.

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