Cobalamin-dependent methionine synthase

RUMA V. BANERJEE AND ROWENA G. MATTHEWS

Biophysics Research Division and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109, USA

ABSTRACT

Cobalamin-dependent methionine synthase catalyzes the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine, producing tetrahydrofolate and methionine. Insufficient availability of cobalamin, or inhibition of methionine synthase by exposure to nitrous oxide, leads to diminished activity of this enzyme. In humans, severe inhibition of methionine synthase results in the development of megaloblastic anemia, and eventually in subacute combined degeneration of the spinal cord. It also results in diminished intracellular folate levels and a redistribution of folate derivatives. In this review, we summarize recent progress in understanding the catalysis and regulation of this important enzyme from both bacterial and mammalian sources. Because inhibition of mammalian methionine synthase can restrict the incorporation of methyltetrahydrofolate from the blood into cellular folate pools that can be used for nucleotide biosynthesis, it is a potential chemotherapeutic target. The review emphasizes the mechanistic information that will be needed in order to design rational inhibitors of the enzyme. — BANERJEE, R. V.; MATTHEWS, R. G. Cobalamin-dependent methionine synthase. *FASEB J.* 4: 1450–1459; 1990.

Key Words: methionine synthase · cobalamin · vitamin B_{12} · nitrous oxide · methyltetrahydrofolate

METHIONINE SYNTHASE (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13) catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH_3-H_4folate)[1] to homocysteine, generating H_4folate and methionine as shown in *Eq. 1.*

$$CH_3-H_4\text{folate} + \text{Homocysteine} \rightarrow H_4\text{folate} + \text{Methionine} \quad (1)$$

In prokaryotes that synthesize methionine de novo, this reaction represents the terminal step in methionine biosynthesis. Methionine synthase is thus poised at the point of convergence of two major biosynthetic pathways: the tetrahydrofolate-dependent pathway for biosynthesis of methyl groups and the homocysteine biosynthetic pathway. Mammals, on the other hand, are unable to synthesize homocysteine de novo; rather they use methionine synthase to regenerate methionine from homocysteine to provide one-carbon units for S-adenosylmethionine (AdoMet)-dependent methylation reactions.

Escherichia coli synthesize two distinct proteins with methionine synthase activity. The *metH* gene product is a cobalamin-dependent enzyme (EC 2.1.1.13) that uses monoglutamate as well as polyglutamate forms of CH_3-H_4 folate as substrates, and must be activated for catalysis by a reductive methylation involving AdoMet and an electron donor (1, 2). The *metE* gene product is a cobalamin-independent form of the enzyme (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, EC 2.1.1.14) that has a strict requirement for the polyglutamate form of the substrate CH_3-H_4folate (3). In vitro assays can easily distinguish between the two activities; MetE requires magnesium and phosphate ions for activity (3), whereas MetH has an absolute requirement for AdoMet and a reducing system (2). In addition, the standard assay for cobalamin-dependent methionine synthase includes the monoglutamate form of CH_3-H_4folate and thereby excludes reaction of MetE.

Although the prokaryotic cobalamin-dependent methionine synthase has been studied extensively (reviewed in refs 4 and 5), the mammalian enzyme is much less well characterized. The *metH* gene from *E. coli* has been cloned and its gene product overexpressed (6). The mammalian enzyme appears very similar in its catalytic properties to the *metH* gene product from *E. coli.* Thus, the mammalian enzyme also contains cobalamin (7) and is competent to react with the monoglutamate form of CH_3-H_4folate. It, too, is isolated in an inactive form and requires AdoMet-dependent reductive methylation for activation (7). The apparent similarity of the catalytic mechanisms of the cobalamin-dependent enzymes from bacterial and mammalian sources, and the ready availability of the bacterial MetH protein, make this enzyme an attractive choice for mechanistic studies.

In this review, we begin by presenting a brief overview of methionine synthase and its role in folate

[1]Abbreviations: AdoMet, S-adenosyl-L-methionine; CH_3-H_4folate, N^5-methyltetrahydrofolate; CH_2-H_4folate, N^5,N^{10}-methylenetetrahydrofolate; AdoHCy, S-adenosyl-L-homocysteine; kDa, kilodalton; HCy, homocysteine; $CH=H_4$folate, N^5,N^{10}-methenyltetrahydrofolate; $CHO-H_4$folate; N^{10}-formyltetrahydrofolate; H_2folate, dihydrofolate; bp, base pairs.

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metabolism and then consider the mammalian enzyme as a potential target for chemotherapeutic intervention. The inactivation of enzyme from both mammalian and bacterial sources by nitrous oxide (N_2O) is discussed. Studies of the nucleotide sequence analysis of the *metH* gene and structure-function studies of the encoded protein are summarized. Finally, salient chemical problems associated with catalysis of this methyl transfer reaction are discussed within the framework of a possible mechanism of catalysis.

OVERVIEW OF METHIONINE SYNTHASE AND ITS ROLE IN FOLATE METABOLISM

The pioneering studies by du Vigneaud and co-workers (8) and by Bennett (9) demonstrated that dietary requirements for methionine in the rat could be replaced by supplying homocysteine and cyanocobalamin. Studies from the laboratories of Buchanan, Kisliuk, and Woods (reviewed in refs 4 and 5) demonstrated the presence of a cobalamin cofactor in methionine synthase purified from both *E. coli* and pig liver.

The structure of the cobalamin cofactor of methionine synthase is shown in **Fig. 1.** The cobalt ion in cobalamin is coordinated by four coplanar nitrogen ligands in a reduced tetrapyrrole structure, the corrin ring. Two additional coordination sites exist: the upper and lower axial positions that lie above and below the plane of the ring, respectively. In methylcobalamin, a methyl group is bonded to cobalt in the upper axial position, and the

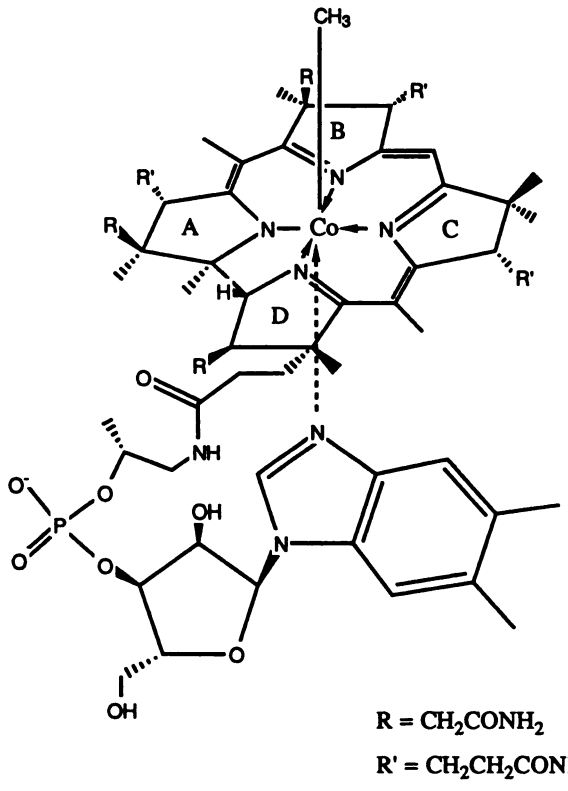

Figure 1. Structure of the cobalamin cofactor of methionine synthase. The structure drawn is that of methylcobalamin, with the methyl group occupying the upper axial coordination site of cobalt.

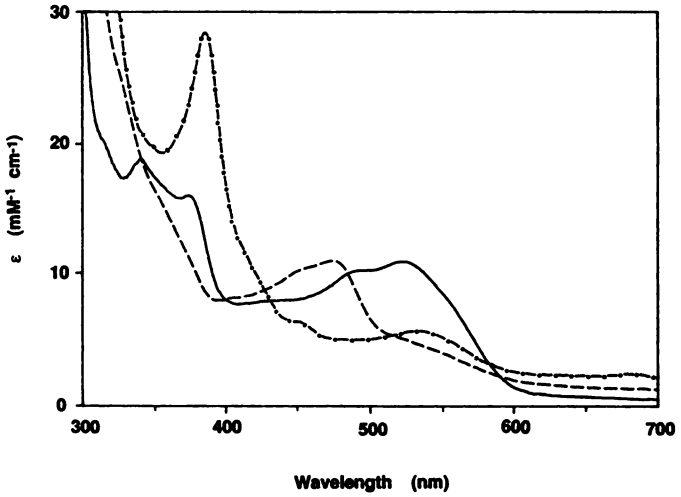

Figure 2. Electronic absorbance spectra of enzyme-bound cobalamin at different states of reduction and of methylated enzyme. —, methylcobalamin; ----, cob(II)alamin; -·-·-·-, cob(I)alamin.

lower axial position is occupied by a nitrogen of the dimethylbenzimidazole nucleotide substituent of the corrin ring. During turnover, the cobalamin cofactor of methionine synthase shuttles between methylcobalamin and cob(I)alamin (which contains a pair of electrons in the dz^2 orbital oriented perpendicularly to the plane of the corrin ring). The enzyme-bound cob(I)alamin can be oxidized to cob(II)alamin, with a single electron in the dz^2 orbital, or to cob(III)alamin. The different forms of cobalamin can readily be distinguished by their ultraviolet and visible electronic absorbance spectra (**Fig. 2**).

The cobalamin-dependent enzyme from *E. coli* B was extensively purified by Taylor and Weissbach (10) and was purified to homogeneity from *E. coli* K-12 by Fujii and Huennekens (11). Studies from these two laboratories, and from those of Kisliuk and Woods (reviewed in refs 4, 5), suggested the catalytic mechanism shown in **Fig. 3**, in which the enzyme cycles during turnover between methylcobalamin and cob(I)alamin. The participation of methyl cobalamin as a catalytic intermediate was demonstrated by radiolabel tracer studies (12). Cob(I)alamin was formed by demethylation of methylated enzyme under anaerobic conditions, as deduced by characteristic changes in the visible electronic absorbance spectrum when homocysteine was added to the enzyme-bound methylcobalamin (13, 14).

Concomitant studies of the enzymes involved in the conversion of the β-carbon of serine into the methyl group of AdoMet (**Fig. 4**) elucidated the regulation of AdoMet biosynthesis. Serine hydroxymethyltransferase catalyzes the transfer of the β-carbon of serine to H_4folate to generate CH_2-H_4folate and glycine. Methylenetetrahydrofolate reductase (NADPH) (EC 1.5.1.20) catalyzes the reduction of CH_2-H_4folate to CH_3-H_4folate. The overall NADPH-linked reduction of CH_2-H_4folate to CH_3-H_4folate is irreversible (15) and commits folate-bound one-carbon units to use for the methylation of homocysteine. Methionine synthase catalyzes the transfer of the methyl group of CH_3-H_4folate to homocysteine

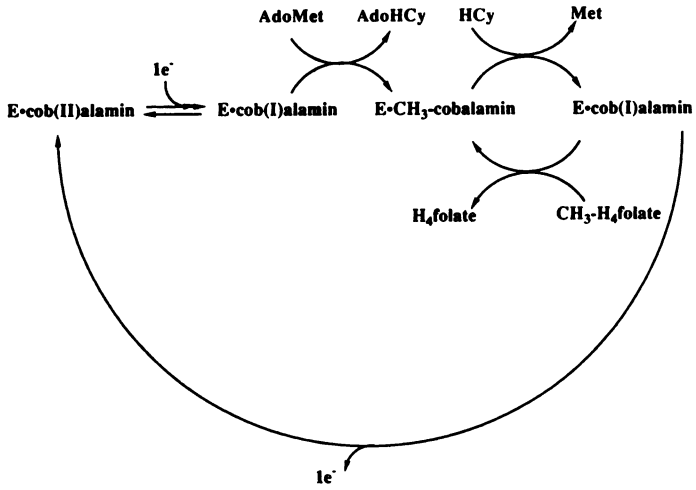

Figure 3. Schematic mechanism for activation and catalysis of methionine synthase. The cob(II)alamin form of the enzyme is inactive. In vitro activation involves a reductive methylation with AdoMet as the methyl donor. The methylated enzyme, once formed, is catalytically active and cycles between cob(I)alamin and methylcobalamin in catalysis, as shown. Occasional oxidation of the cob(I)alamin intermediate to the inactive cob(II)alamin mandates the continuing requirement for the activation system during catalysis.

to form methionine and H_4folate, and methionine can then be used for protein synthesis or converted to AdoMet. In mammals, this pathway is regulated by the AdoMet/AdoHCy ratio, and AdoMet has been shown to be an allosteric inhibitor of methylenetetrahydrofolate reductase from mammalian sources (16, 17). AdoHCy, the product of AdoMet-dependent methylation reactions, blocks the binding of AdoMet to methylenetetrahydrofolate reductase, but does not itself inhibit or activate the enzyme. In *E. coli,* AdoMet, rather than methionine, is also the major regulator of methionine biosynthesis and combines with an aporepressor protein, the *metJ* gene product, to inhibit transcription of enzymes involved in methionine biosynthesis (reviewed in ref 18).

Inhibition of methionine synthase interferes with AdoMet-dependent regulation of the cellular levels of CH_3-H_4folate. Under these circumstances, inadequate intake of dietary methionine leads to a fall in AdoMet concentrations that can no longer be prevented by remethylation of homocysteine. Methylenetetrahydrofolate reductase remains fully active, and cellular folates accumulate as CH_3-H_4folate. These changes result in a cellular methyl trap as first proposed by Noronha and Silverman (19) and by Herbert and Zaluskey (20). Cobalamin deficiency also leads to an accumulation of the CH_3-H_4folate pool at the expense of the other cellular folate pools owing to the decreased activity of methionine synthase. The depletion of CH_2-H_4folate required for thymidylate biosynthesis appears to be the primary cause of megaloblastic anemia associated with either folate or cobalamin deficiency in humans. Megaloblastic anemia is not associated with folate or cobalamin deficiency in other mammals.

Inhibition of methionine synthase leads not only to redistribution of cellular folate derivatives, but also to a decline in the total intracellular folate level. The major circulating form of folate is CH_3-H_4folate, and methionine synthase activity is required to convert this derivative to forms that can be used for nucleotide biosynthesis (15). Moreover, CH_3-H_4folate is a poor substrate for folylpolyglutamate synthase (21), the enzyme that catalyzes sequential poly-γ-glutamylation of intracellular folate derivatives to generate folylpolyglutamates, which are the predominant intracellular forms of folic acid. The polyglutamate forms of folic acid are essential for intracellular retention of the vitamin. Hence a shift in the cellular folate pool to methyl derivatives results in a shift toward shorter-chain forms that can traverse the cell membrane more readily and lead to an intracellular folate deficiency.

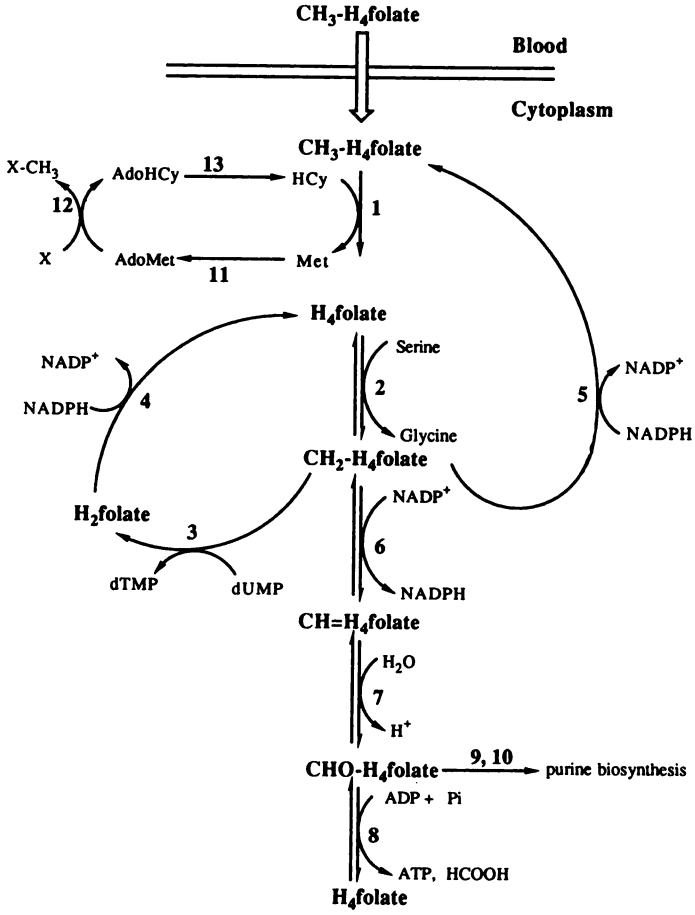

Figure 4. Outline of the major tetrahydrofolate-dependent biosynthetic pathways. The enzymes involved are: 1, methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase); 2, serine hydroxymethyltransferase; 3, thymidylate synthase; 4, dihydrofolate reductase; 5, methylenetetrahydrofolate reductase; 6, methylenetetrahydrofolate dehydrogenase; 7, methenyltetrahydrofolate cyclohydrolase; 8, formyltetrahydrofolate synthetase; 9, glycineamide ribonucleotide transformylase; 10, aminoimidazolecarboxamide ribonucleotide transformylase; 11, adenosylmethionine synthetase; 12, adenosylmethionine-dependent methyltransferases; and 13, adenosylhomocysteine hydrolase.

Methionine synthase thus may be viewed as a crucial player in folate metabolism, poised at the point of entry of folates into the cell and responsible for converting the vitamin from CH_3-H_4folate into a more widely useful form that can participate in nucleotide biosynthesis. Methionine, the other product of the methionine synthase-catalyzed reaction, is an essential amino acid in mammals, and serves in protein synthesis and as the precursor for AdoMet. The latter is a crucial methyl group donor involved in the biosynthesis of a variety of compounds including creatine, phosphatidylcholine, and epinephrine. AdoMet shortage in cells lacking active methionine synthase may be alleviated by the provision of exogenous methionine, whereas conversion of CH_3-H_4folate to H_4folate would remain compromised. Methylenetetrahydrofolate reductase activity should remain inhibited under this regime, preventing the accumulation of cellular folylpolyglutamates as CH_3-H_4folate.

The relationship between impaired methionine synthase activity and diminution of cellular folate pools should make it an attractive chemotherapeutic target. Rapidly dividing cells should have an elevated requirement for uptake of folate from the blood, and may be particularly hard hit by inhibition of methionine synthase. Several enzymes involved in folate metabolism, including thymidylate synthase and dihydrofolate reductase, have already been realized as chemotherapeutic targets. Inhibition of methionine synthase, with its potential for depleting the folate pool with consequent inhibition of nucleotide biosynthesis, holds a similar promise. As yet no antineoplastic drug directed against methionine synthase is available.

INACTIVATION OF METHIONINE SYNTHASE BY NITROUS OXIDE

Exposure of humans to nitrous oxide (N_2O), a commonly used anesthetic agent, leads to inactivation of methionine synthase (22). Prolonged exposure leads to the development of megaloblastic anemia in humans but not in other mammals (23), and in cases of repeated exposure may even result in the neurological symptoms characteristic of pernicious anemia or cobalamin deficiency, such as subacute combined degeneration of the spinal cord (24). Treatment of rodents with N_2O has been shown to lead to a rapid decrease in the levels of methionine synthase activity in liver homogenates (25–27). Recovery of activity required several days after discontinuation of N_2O treatment and presumably was linked to synthesis of new enzyme. Long-term administration of N_2O to monkeys resulted in subacute combined degeneration of the spinal cord, and these symptoms could be alleviated by methionine supplementation of the diet (28). Since the effect of nitrous oxide appears to be specific for methionine synthase, an understanding of the events associated with this inactivation might point the way to the design of other inhibitors of methionine synthase.

Model studies of the interaction of the cob(I)alamin in aqueous solution with N_2O have established that cob(I)alamin is oxidized and N_2 is liberated (29, 30), presumably according to *Eq. 2* and *3*.

$$\text{cob(I)alamin} + N_2O + 2H^+ \rightarrow \text{cob(III)alamin} + N_2 + H_2O \quad (2)$$

$$\text{cob(III)alamin} + \text{cob(I)alamin} \rightarrow 2\text{cob(II)alamin} \quad (3)$$

As these experiments were conducted in the presence of an excess of cob(I)alamin, the formation of cob(III)alamin was not directly demonstrated but was implied from the observed formation of cob(II)alamin.

The similarity of cobalamin-dependent methionine synthase enzymes from prokaryotic and eukaryotic sources extends to their susceptibility to inhibition by N_2O. Purified methionine synthase preparations from *E. coli* B and from pig liver have been used to demonstrate the in vitro inactivation of the enzyme by N_2O (31). Inactivation occurred only in the presence of all components required for turnover. The ratio of moles of methionine formed to moles of enzyme inactivated was approximately 3900 for the bacterial enzyme. Presumably because of the requirement for multiple turnovers per inactivation event, inactivation occurred only in very dilute enzyme solutions ($\sim$6 nM enzyme), so that absorbance changes associated with enzyme inactivation could not be monitored directly. When the inactivated enzyme was reconcentrated from the assay solution so that its absorbance properties could be examined, significant loss of the cobalamin was observed in the N_2O-treated enzyme as compared to control N_2-treated enzyme, but both samples retained the spectrum of cob(II)alamin. Based on the model studies, it was postulated that N_2O intercepts enzyme-bound cob(I)alamin that is generated transiently during turnover, with formation of cob(II)alamin and hydroxyl radical as shown in *Eq. 4*. Generation of a rogue hydroxyl radical at the active site could explain the observed irreversible loss of enzyme activity.

$$\text{cob(I)alamin} + N_2O + H^+ \rightarrow \text{cob(II)alamin} + N_2 + OH\cdot \quad (4)$$

Studies aimed at directly monitoring the reaction of enzyme bound cob(I)alamin with N_2O are in progress in our laboratory.

CLONING AND SEQUENCE ANALYSIS OF THE METH GENE

In *E. coli*, methionine synthase catalyzes the terminal step in the de novo biosynthesis of methionine. Under conditions of aerobic growth the bacteria are unable to synthesize cobalamin, although the MetH apoenzyme is synthesized. In the absence of methionine or cobalamin in the medium, aerobic cell growth requires the MetE protein. When coablamin is added to the medium, the MetH holoenzyme is formed and MetE synthesis is repressed. A methionine-requiring nutritional auxotroph of *E. coli* (RK4536: *metH*$^-$, *metE*$^-$) was used to isolate a recombinant clone that was prototrophic for methionine in the presence of cobalamin (32, 33). The identity

of the cloned *metH* gene was confirmed by nucleotide sequence analysis, maxicell expression, and enzyme activity assays. The sequence of both strands was completely determined, including 220 bp upstream and 180 bp downstream of the protein coding sequence (6). The open reading frame encodes 1,124 amino acids with a predicted molecular mass of 123,640 daltons, within 9% of the value given by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by gel filtration (34). More recently, the nucleotide sequence of the *metH* gene from *E. coli* K-12 has been determined (35) and a 3600-bp open reading frame that encodes a peptide with a predicted molecular weight of 132,628 was found. The amino acid sequence deduced by the two groups diverges at position 3337. The difference appears to result from omission of a G from the sequence TGGGG starting at position 3334, which results in a stop codon at position 3374 of the shorter open reading frame. The validity of the COOH-terminal extension was established by the expression of β-galactosidase activity by a *metH-lacZ* fusion located 190 bp downstream of the putative stop codon at position 3374 (35).

Comparison of the deduced amino acid sequences of the *E. coli metH* gene and the proximal third of the *Salmonella metH* gene (36) revealed a remarkable similarity, with only 21 of the 371 amino acids being different. In contrast, comparison with all other sequences in the NBRF and GenBank databases failed to identify any significant homologies. Both *metH* and *metE* genes have been shown to be subject to positive regulation by the *metR* gene product, a *trans*-acting transcriptional activator (37, 38). Although no extensive regions of homology are apparent in the sequences of the *metE* and *metH* control regions of *Salmonella typhimurium*, a short region of interrupted dyad symmetry was found (36). Mutations in this region of the *metE* gene disrupt the normal regulation of the *metE* gene by the *metR* gene product (36). The *metR* gene product has been shown to bind to an upstream region in the *E. coli metE* gene (39). Upstream of the *E. coli metH* gene a similar sequence has been found (35, 40). The four sequences are shown in **Fig. 5.** The finding of only partial homology between the control regions of the two genes is not surprising, since they are regulated to vastly different extents by the *metR* gene product (36). Hence, although the range of activation of the *metE* gene is nearly 100-fold, that of the *metH* gene is only five- to sixfold. The observation that the dyadic symmetry is more extensive in the two *metE* sequences than in the analogous *metH* sequences is consistent with the differential effect of the *metR* gene on transcriptional regulation of these two genes.

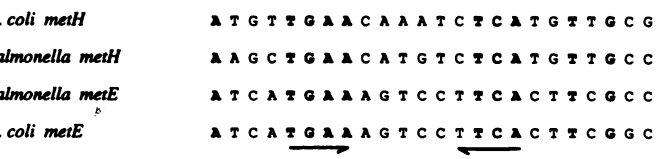

Figure 5. Comparison of the upstream sequences of the *metE* and *metH* genes that are presumably involved in regulation by *metR*. The regions compared show the interrupted dyad symmetry sequence; mutations in this region disrupt the normal regulation of the *metE* gene by the *metR* gene product in *Salmonella* (36). Residues conserved in all four genes are shown in bold type. Each of the sequences is upstream of the corresponding -35 region of its gene. The direction of transcription of all three genes is to the right. The papers from which these sequences are drawn are noted in the text.

THE COBALAMIN-BINDING DOMAIN: AN α/β STRUCTURE?

Since primary sequence comparison of MetH with other folate- and cobalamin-binding proteins failed to reveal any significant relationship, we sought to use limited proteolysis to identify a fragment of the enzyme containing the cobalamin-binding site (6). Tryptic digestion of the native recombinant enzyme resulted in the formation of two daughter fragments of 95 and 35 kDa, and was accompanied by loss of activity. NH_2-terminal sequence analysis of the 35-kDa fragment established its position at the COOH-terminal end of the protein. Although the 35-kDa fragment was fairly stable over the time course of the digestion, the 95-kDa peptide was cleaved to form 68- and 28-kDa peptides. Separation of the peptides on a native nondenaturing gel resulted in the identification of a pink fragment that retained the cobalamin-binding site and had a molecular mass of 28,000. NH_2-terminal analysis of the 28-kDa fragment established the limits of the cobalamin-binding domain. The hypersensitive site at the COOH-terminus of the cobalamin-binding domain, between residues 900 and 901, lies in one of the most hydrophilic internal regions of the protein as predicted by the method of Kyte and Doolittle (41). The 28-kDa fragment is immediately upstream of the 35-kDa fragment, and is characterized by the prevalence of hydrophobic residues.

Comparison of the deduced sequence of the 28-kDa fragment with those of other soluble cobalamin-binding proteins such as BtuR (a cobalamin-binding protein from *E. coli* implicated in the metabolism of adenosylmethionine) (42), MutA and MutB (the two subunits of methylmalonyl CoA mutase from *Propionibacterium shermanii*) (43), and EaIA and EaIB (the two subunits of ethanolamine ammonia lyase from *Salmonella typhimurium*) (L. P. Faust, J. A. Connor, D. M. Roof, J. A. Hoch, and B. M. Babior, unpublished results), failed to detect any significant homologies. However, predicted secondary structure comparisons of the four proteins revealed a striking similarity in the pattern and spacing of alternating α-helices and β-sheets spanning a length of about 100 amino acids in the deduced sequences of each of the four proteins, as shown in **Fig. 6.** In the absence of a crystal structure for any member of the cobalamin-binding protein family, it was postulated that the cobalamin pocket may contain an α/β type of structure (6). The alternation of α-helices and β-sheets is suggestive of the Rossman fold motif that is involved in binding nucleotides (44). The structure of cobalamin contains dimethylbenzimidazole in an unusual nucleotide. A comparison of the

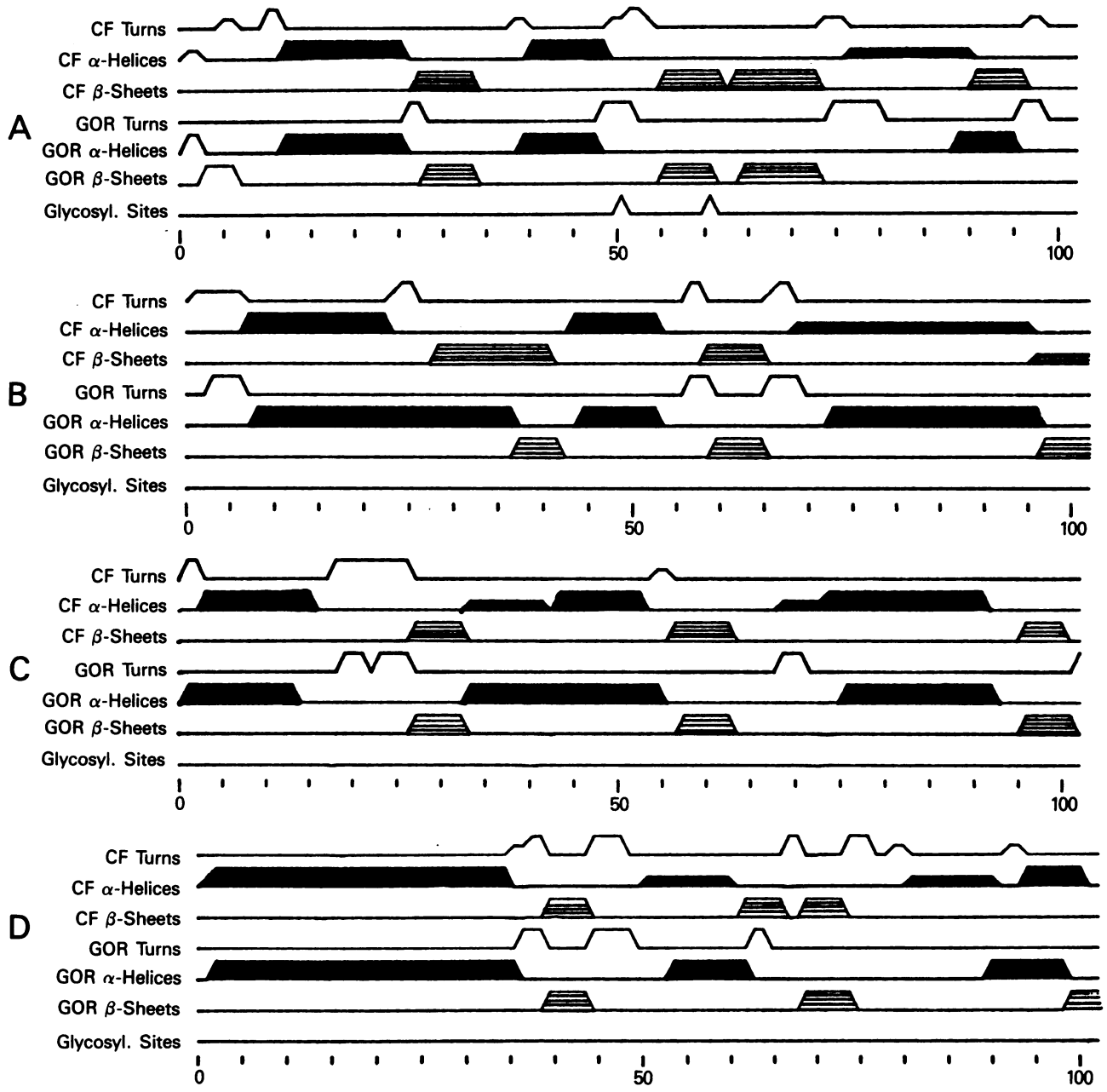

Figure 6. Predicted secondary structure comparisons of segments of four cobalamin-binding proteins. A, Residues 800-900 of MetH (6); B, residues 96-196 of BtuR (42); C, residues 505-605 of MutB (43); D, residues 110-210 of EalB (B. M. Babior, personal communication). CF, Chou-Fasman secondary structure prediction; GOR, Garnier-Osguthorpe-Robson secondary structure prediction. The thickness of the black bars is proportional to the strength of the prediction.

benzimidazole-ribofuranosyl portion of cobalamin with the adenine ribose of NAD bound to crystalline malate dehydrogenase revealed a good alignment of the two molecules with a calculated root mean square distance between corresponding atoms of 1.05 Å (45). The region of identified secondary structural homology in the cobalamin-binding proteins may be involved in docking the dimethylbenzimidazole nucleotide.

Conservation of secondary and tertiary structure in binding domains in the absence of primary sequence conservation is not without precedent. Hence, in the globins and cytochromes, peptides with poor primary sequence homology fold into proteins of striking architectural similarity. In the globins, for instance, the pocket in which the heme cofactor binds is created by the assembly of eight α-helices. Only 5 of the 116 positions that are involved in conserved interactions with the cofactor are identical in all globins, and even they are not contiguous in the primary sequence (46). This illustrates the difficulty in predicting the relatedness of the globin family members from primary sequence comparisons alone.

ACTIVATION OF METHIONINE SYNTHASE AND ITS CATALYTIC MECHANISM

The properties of the *E. coli* B enzyme that has been purified to homogeneity (34) are similar to those de-

scribed for the partially purified enzyme from the same source (10) and for the homogeneous wild-type (11) and recombinant (6) enzymes from *E. coli* K-12. The enzyme is monomeric, and contains 1 mol of cobalamin per mol of enzyme. Electron spin resonance (EPR) spectroscopy of the enzyme as isolated in its inactive form reveals the presence of cob(II)alamin (34), which is consistent with the electronic absorption spectrum of the enzyme. The EPR signal disappears upon reductive methylation, as expected for the conversion of paramagnetic cob(II)alamin to diamagnetic methylcobalamin (Fig. 3). In addition to cobalt, the *E. coli* B enzyme was found to contain 1 mol of copper per mol of enzyme-bound cobalamin (34). The copper was tightly bound and EPR silent in the native enzyme, but became detectable when the enzyme was denatured. The copper bound to the enzyme is in the Cu^{+1} oxidation state as judged by X-ray absorption edge spectroscopy (Clark, K., Penner-Hahn, J., Banerjee, R. V., and Matthews, R. G., unpublished results). The recombinant enzyme from *E. coli* K-12 lacks stoichiometric copper (it contains about 0.1 mol copper per mol cobalamin) although its turnover number under V_{max} conditions is comparable to that of the wild-type *E. coli* B enzyme. These results lead us to conclude that copper does not play a crucial catalytic role.

Methionine synthase has also been purified to homogeneity from human placental tissue (47) and found to contain two equivalents of iron per mol of enzyme-bound cobalamin. The role of iron in this enzyme is as yet unknown. The homogeneous enzyme from human placenta had a specific activity of 0.014 μmol min^{-1} mg $protein^{-1}$, measured at 37°C, which would correspond to a turnover number of 2.2 min^{-1}. In contrast, a turnover number of 670 min^{-1} was estimated for partially purified enzyme from pig liver (7), and a specific activity of 0.9 μmol min^{-1} mg $protein^{-1}$ at 37°C was measured for the partially purified pig kidney enzyme (48). Thus there is concern that the enzyme isolated from human placenta (47) was grossly inactive. The turnover number of 670 min^{-1} estimated on the basis of cobalamin determinations in partially purified enzyme fractions (7) is quite comparable to the turnover number of the enzyme from *E. coli* ($\sim$1300 min^{-1} at 37°C) (34).

The overall reaction catalyzed by methionine synthase is a transmethylation from CH_3-H_4folate to homocysteine, as shown in *Eq. 1,* and has an equilibrium constant of 10^5 in favor of products (49). Under in vitro assay conditions, the reaction displays an absolute requirement for catalytic amounts of AdoMet and a reducing system (1, 2). Inactive cob(II)alamin is primed for turnover by a reductive methylation reaction, as shown in Fig. 3. The methylated enzyme so generated is catalytically competent and mediates the transfer of a methyl group from CH_3-H_4folate to homocysteine during turnover. However, about once in every 100 turnovers, the enzyme is disabled by an adventitious oxidation of cob(I)alamin (50), and needs to be reactivated for turnover by the reducing system and AdoMet.

A variety of different activation systems have been employed with the enzyme from *E. coli.* The standard assay includes dithiothreitol and aquocobalamin in phosphate buffer as the source of electrons. The chemical reductants can be substituted by NADPH and two flavoproteins from *E. coli,* designated the R and F proteins (11), that have properties analogous to ferredoxin: NADPH oxidoreductase and flavodoxin, respectively. With the physiological reducing system, the ultimate source of electrons is NADPH, an obligate two-electron donor. Although the R flavoprotein undergoes a facile two-electron reduction by NADPH, the second protein in the electron transfer chain, the F protein, stabilizes the one-electron reduced semiquinone state (50). Hence the role of the two flavoproteins in this electron wire between NADPH and methionine synthase is presumably to mediate conversion from two to one electron transfer. With the chemical reducing system, aquocobalamin may assume a similar role in bridging the gap between dithiothreitol, a two-electron donor, and methionine synthase, a one-electron acceptor. In addition, or alternatively, aquocobalamin plays the role of an oxygen scrubber, providing semianaerobic conditions for assays conducted with initially aerobic solutions. Apoenzyme from bacterial sources is inactive in assays containing aquocobalamin and requires methylcobalamin for holoenzyme formation (51).

Conversion of cob(II)alamin to cob(I)alamin is a particularly challenging reduction in the cellular milieu because the midpoint potential for this redox couple is one of the lowest found in biological systems (**Fig. 7**). The cob(II)alamin/cob(I)alamin couple of the aqueous cofactor at pH 7 was determined to be -610 mV vs. the standard hydrogen electrode (52). The reduction potential for the methionine synthase-bound cob(II)alamin/cob(I)alamin couple was found to be -526 mV (53). Hence, although the binding of the cofactor to the protein does render the reduction somewhat easier, reduction remains a thermodynamically unfavorable reaction with the reducing partners of methionine synthase in in vitro assays, viz. flavodoxin or dithiothreitol, as shown in Fig. 7.

An endergonic reduction could in principle be driven by a large free energy decrease accompanying methylation of the reduced cobalamin, if the two reactions were coupled. Evidence for such a mechanism for reductive methylation of methionine synthase has recently been obtained (53). The highly exergonic methyl transfer from AdoMet to cob(I)alamin could be used to drive a highly endergonic reduction of the cob(II)alamin redox center of the enzyme at potentials as high as -82 mV. From the shift in the apparent standard potential for the cob(II)alamin/cob(I)alamin couple in the presence of AdoMet, $\Delta G^{o'}$ for the methyl transfer was estimated to be greater than -9 kcal per mol. In contrast, $\Delta G^{o'}$ for the CH_3-H_4folate-dependent methylation was calculated to be -0.09 kcal per mol, and the apparent standard potential for the cob(II)alamin/cob(I)alamin couple in the presence of CH_3-H_4folate is only shifted to -450 mV. These results elucidate the enigmatic requirement for AdoMet during in vitro activation,

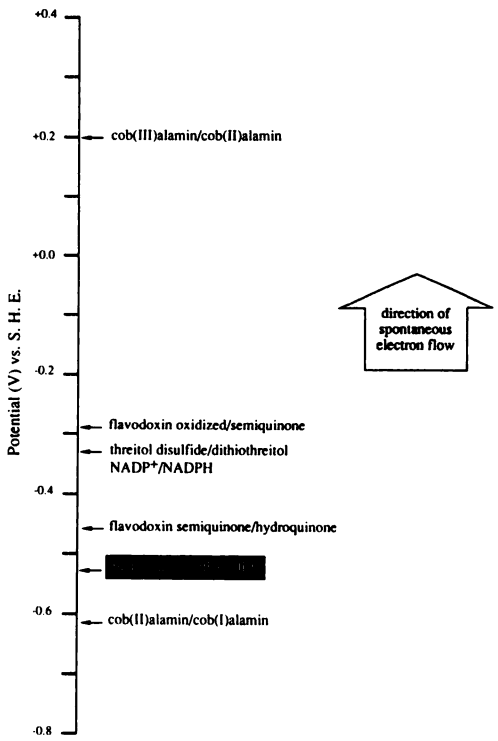

Figure 7. Redox potential ladder demonstrating the thermodynamic difficulty of reducing cob(II)alamin in the cellular milieu, and the rationale for employing AdoMet vs. CH_3-H_4folate to couple methylation of cob(I)alamin to reduction of cob(II)alamin to cob(I)alamin. For a two electron transfer at 25°C, a 30 mV potential difference corresponds to an ~10-fold difference in concentrations of the two species at equilibrium, and for a one electron transfer, a 60 mV potential difference corresponds to an ~10-fold difference in concentrations of the two species at equilibrium. All potentials shown are vs. the standard hydrogen electrode and are standard potentials at pH 7.

despite the ready reaction of CH_3-H_4folate with cob(I)alamin during catalytic turnover. The equilibrium constants for the two transmethylations differ by more than 10^9; hence AdoMet, but not CH_3-H_4folate, is able to drive reduction of cob(II)alamin at ambient potentials during aerobic growth and under in vitro assay conditions.

There is no evidence for the presence of a pyridine nucleotide-dependent activation system analogous to the R and F protein in pig liver cells. However, several thiols, such as homocysteine, cysteine, and dithiothreitol, are capable of supporting methionine synthase activity in the absence of added cobalamin (54). Partially purified enzyme is associated with thiol oxidase activity (54), as shown in *Eq. 5.*

$$\text{Dithiothreitol} + O_2 \rightarrow \text{Threitol disulfide} + H_2O_2 \qquad (5)$$

It has been postulated that the thiol oxidase activity is an intrinsic property of the mammalian enzyme, and that thiols may serve as the physiological reductants for the mammalian enzyme (54). A group of patients with severe megaloblastic anemia and homocystinuria (but no methylmalonic aciduria) have been described (55), and their symptoms appear to be associated with a genetic defect in cobalamin metabolism (cblE). Cells from these patients had normal methionine synthase activity under standard assay conditions, but the enzyme activity was greatly reduced when measured with suboptimal concentrations of thiol reducing agents. Significantly, the patient's fibroblasts were insensitive to exposure to nitrous oxide, suggesting that methionine synthase was inactive in these cells. Although the identity of the loci affected by the cblE mutation is unknown, it is speculated that it affects a reducing system analogous to that in *E. coli.* Characterization of the mutation should provide a powerful tool for understanding the mechanism of activation of the human methionine synthase.

Once methylcobalamin enzyme is formed, catalysis proceeds with alternation of demethylation and methylation as shown in Fig. 3. The stereochemistry of the overall methyl transfer reaction from CH_3-H_4folate to homocysteine was investigated using CH_3-H_4folate that was chiral at the 5-methyl position by virtue of containing deuterium and tritium (56). The reaction was found to proceed with retention of configuration at the transferred carbon, as expected for two consecutive methyl transfers from CH_3-H_4folate to cobalamin and then from methylcobalamin to homocysteine.

We have used stopped-flow kinetic studies to determine the kinetic competence of the cob(I)alamin and methylcobalamin species at 25°C, under rigorously anaerobic conditions (Banerjee, R. V., Frasca, V., Ballou, D. P., and Matthews, R. G., unpublished data). Under such strictly anaerobic conditions, the need for an activation system is obviated because of the reduced susceptibility of cob(I)alamin to oxidation. Hence, the turnover reaction can be studied starting with the methylcobalamin form of methionine synthase in the absence of added AdoMet and aquocobalamin. These experiments were performed with methylated methionine synthase that had been generated by electrochemical reduction in the presence of AdoMet (53). The enzyme was rapidly mixed with either homocysteine or a mixture of homocysteine and CH_3-H_4folate. The formation of cob(I)alamin from methylcobalamin was monitored either by observing the increase in absorption at 390 nm or the decrease in absorption at 520 nm (see Fig. 2). Conversely, the formation of methylcobalamin from cob(I)alamin was followed by monitoring the increase in absorbance at 520 nm. In these experiments, evidence has been obtained that both cob(I)alamin and methylcobalamin are kinetically competent catalytic intermediates.

Although there is good evidence for the intermediacy of methylcobalamin in the methyl group transfer from CH_3-H_4folate to homocysteine, and for the role of cob(I)alamin as a nucleophile, the mechanism of activation of CH_3-H_4folate prior to nucleophilic displacement of the methyl group remains obscure. A simple S_N2 displacement at the α-carbon of a tertiary amine would constitute a chemical reaction without precedent, albeit cob(I)alamin is an excellent nucleophile. As shown in **Fig. 8,** the tertiary amine could be activated by two-electron oxidation giving rise to a quaternized sp^2 nitrogen (**structures** 1 and 2), by one electron oxi-

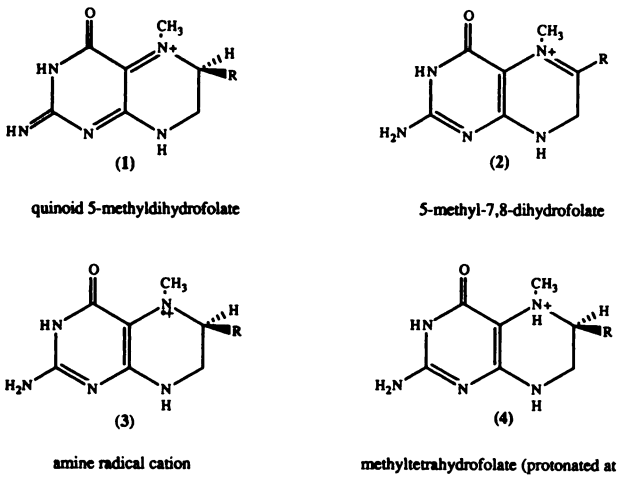

Figure 8. Possible activated forms of CH_3-H_4folate.

dation to generate the amine radical cation (structure 3), or by quaternization via protonation at N^5 (structure 4). Formation of **structures** 1, 2, or 3 would require an as-yet-unidentified high potential electron acceptor on the enzyme. Whatever the mode of activation, an understanding of the structure of the activated species (i.e., whether N^5 is trigonal or tetrahedral; charged or neutral) would have an important bearing on the rational design of CH_3-H_4folate analogs as inhibitors of the enzyme.

In summary, although the availability of large amounts of the bacterial methionine synthase from *E. coli* K-12 has led to advances in our understanding of the activation and turnover mechanisms, studies with the mammalian enzyme have lagged behind. Although the enzymes from the two sources share many common properties, the activation systems appear to be different. In particular, it is not known what factors are required for reductive activation of the mammalian enzyme in vivo. Nor is the mode of activation of CH_3-H_4folate preceding displacement of the methyl group understood. An understanding of the reaction mechanism and solution of the crystal structure of methionine synthase holds promise for the rational design of inhibitors with potential antineoplastic activity. FJ

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