Cobalamin-dependent methionine synthase: the structure of a methylcobalamin-binding fragment and implications for other B_{12}-dependent enzymes

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Cobalamin-dependent methionine synthase is a large enzyme composed of structurally and functionally distinct regions. Recent studies have begun to define the roles of several regions of the protein. In particular, the structure of a 27 kDa cobalamin-binding fragment of the enzyme from *Escherichia coli* has been determined by X-ray crystallography, and has revealed the motifs and interactions responsible for recognition of the cofactor. The amino acid sequences of several adenosylcobalamin-dependent enzymes, the methylmalonyl coenzyme A mutases and glutamate mutases, show homology with the cobalamin-binding region of methionine synthase and retain conserved residues that are determinants for the binding of the prosthetic group, suggesting that these mutases and methionine synthase share common three-dimensional structures.

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Introduction

Cobalamin-dependent methionine synthases are key enzymes in the one-carbon metabolism of mammals and microorganisms. Impairment of enzyme activity, either as a result of cobalamin (B_{12}) deficiency [1,2] or following exposure to nitrous oxide [3–6], results in megaloblastic anemia and spinal cord degeneration in humans.

The best characterized methionine synthase is the *metH* gene product from *Escherichia coli*, which has been overexpressed and purified to homogeneity [7]. The enzyme uses methylcobalamin as an intermediate in the transfer of a methyl group from methyltetrahydrofolate (CH_3-H_4 folate) to homocysteine, forming H_4 folate and methionine. The cobalamin is alternately demethylated by homocysteine and methylated by CH_3-H_4 folate:

$$\text{E·methylcobalamin} + \text{homocysteine} \rightarrow \text{E·cob(I)alamin} + \text{methionine} \quad (1)$$

$$\text{E·cob(I)alamin} + CH_3\text{-}H_4 \text{ folate} \rightarrow \text{E·methylcobalamin} + H_4 \text{ folate} \quad (2)$$

A diagram of the catalytic cycle is shown in Fig. 1. In methylcobalamin, the methyl group is directly bonded to the cobalt of cobalamin, and during the catalytic cycle this carbon–cobalt bond is broken and then reformed. As implied by equations 1 and 2, the carbon–cobalt bond is formally cleaved heterolytically, with transfer of a methyl carbocation. The enzyme-bound cob(I)alamin species has been shown to be a kinetically competent intermediate in catalysis [8]. The free cob(I)alamin species is highly reactive, and serves both as an excellent nucleophile [9], and as a strong reductant [10].

The reactive cob(I)alamin intermediate occasionally undergoes oxidation, to form an inactive cob(II)alamin enzyme species. *In vitro*, this oxidation occurs once in every 100–2000 turnovers, depending on the degree of anaerobiosis achieved during turnover, and on the constituents of the assay reaction [11,12•] Reactivation of the cob(II)alamin enzyme requires a reductive methylation to form methylcobalamin enzyme; S-adenosylmethionine (AdoMet) serves as the methyl donor, and the physiological electron donor is believed to be flavodoxin [11,13,14]. AdoMet is required in catalytic amounts and is not a methyl donor in primary turnover (Fig. 1).

Methionine synthase thus catalyzes three different methyl transfers to or from the prosthetic group. S_N2 mechanisms, which have been assumed for the primary reaction cycle, impose strong geometric requirements on the reactants. How then are the substrates homocysteine and CH_3-H_4 folate and the activator AdoMet alternately positioned for methyl transfer? Recent X-ray analysis emphasizes the difficulty of access to the cobalt and its methyl ligand. The structure of the cobalamin-binding domains, described below, shows protein residues capping the top face of the corrin, protecting the methyl

Abbreviations

AdoHcy—*S*-adenosylhomocysteine; **AdoMet**—*S*-adenosylmethionine; **CoA**—coenzyme A; **NAD+**—oxidized nicotinamide adenine dinucleotide; **x**—any amino acid.

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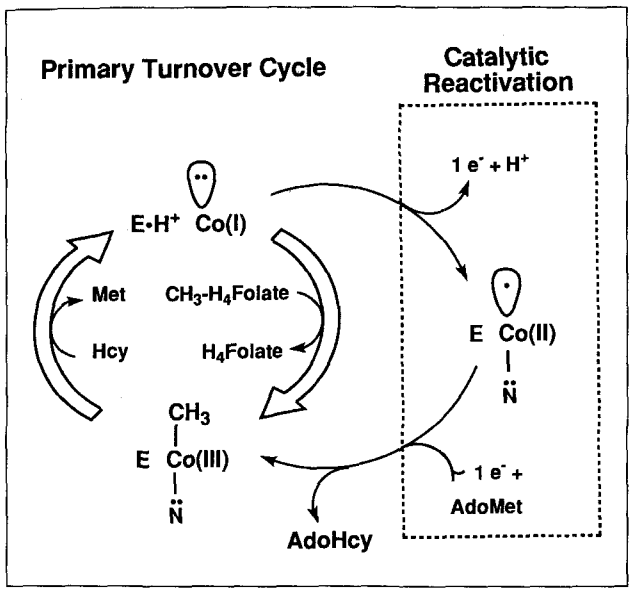

Fig. 1. The catalytic cycle of methionine synthase. During turnover the cobalamin prosthetic group is alternately methylated with a methyl group from N^5-methyltetrahydrofolate, and demethylated with transfer of the methyl group to homocysteine (Hcy). Demethylation results in the formation of enzyme-bound cob(I)alamin, which is assumed to be a four-coordinate species. The electrons remain in the d_{z^2} orbital of cob(I)alamin, which is perpendicular to the plane of the corrin macrocycle. In contrast, the methylated cofactor is six-coordinate, with a nitrogen ligand from His759 occupying the lower axial coordination position [42••]. Enzyme demethylation is depicted as resulting in protonation of a group on the enzyme, and we postulate that the His759–Asp757 pair accepts the proton. The cob(I)alamin prosthetic group is occasionally oxidized during turnover, resulting in the formation of inactive cob(II)alamin. One-electron oxidation of the cofactor is accompanied by proton release [38•]. Return of enzyme to the catalytic cycle requires a reductive methylation of the cofactor, in which the methyl group is provided by *S*-adenosylmethionine (AdoMet) rather than by CH_3-H_4 folate. In *Escherichia coli*, reduced flavodoxin is thought to be the electron donor [11].

group. How the enzyme regulates access to the upper face of the corrin remains an interesting but unresolved issue.

The mammalian enzymes have many properties in common with the better-studied methionine synthase from *E. coli*, suggesting that in many respects the latter enzyme may serve as a model for the mammalian proteins. None of the mammalian genes coding for methionine synthase enzymes has yet been cloned and sequenced, although several mammalian enzymes have been partially purified [15–17]. The porcine enzyme exhibits the same activity per nanomole of cobalamin as the enzyme from *E. coli* [15]; as with the bacterial enzyme, activity is dependent on the presence of AdoMet and a reducing system [18]. Both the mammalian enzyme [18] and the enzyme from *E. coli* [19,20] form methylcobalamin following incubation with AdoMet and a reducing system.

The B_{12}-dependent enzymes that contain adenosylcobalamin rather than methylcobalamin do not form cob(I)alamin during the catalytic cycle, but rather catalyze a homolytic cleavage of the carbon–cobalt bond of adenosylcobalamin to form a 5′-deoxyadenosyl radical and cob(II)alamin. Methylmalonyl coenzyme A (CoA) mutase, the second of the two B_{12}-dependent enzymes found in humans, is an adenosylcobalamin-dependent enzyme [21], as are a number of bacterial amino acid mutases [22,23], ethanolamine ammonia-lyase [24], and ribonucleotide reductase from *Lactobacillus leichmanii* [25]. In these enzymes, the 5′-deoxyadenosyl radical appears to play the role of a radical initiator, abstracting a hydrogen atom either directly from the substrate or from an amino acid residue on the protein. Information from structures and sequences, reported during 1993–1994, has begun to address the resemblances and contrasts between the methyl- and adenosylcobalamin classes of B_{12}-dependent enzymes. A central but open question for enzymologists is how the cobalamin cofactor is influenced by the protein to favor heterolytic cleavage of the carbon–cobalt bond in methionine synthase and homolytic cleavage of the carbon–cobalt bond in adenosylcobalamin-dependent enzymes.

Dissection of cobalamin-dependent methionine synthase into functional and structural units

Cobalamin-dependent methionine synthase is one of the largest polypeptides in *E. coli*, with 1227 residues and a molecular weight of 136 087. To resolve ambiguities in the DNA sequence analyses [7,26], the sequence of the carboxy-terminal portion of the protein was determined from the sizes of peptide fragments using electrospray mass spectrometry [27•]. Initial examination of the amino acid sequence of methionine synthase [7] failed to detect significant homology with any other protein sequences. The starting strategy for defining functional and structural regions of the protein was limited proteolysis of the native holoenzyme. Digestion with trypsin initially cleaves the protein at Arg896 to produce two fragments of 98 and 37 kDa, and amino-terminal sequencing established that the smaller fragment was derived from the carboxyl terminus of the protein. As judged by the red color of the amino-terminal 98 kDa piece, cobalamin remained bound to this fragment. A smaller 28 kDa fragment derived by further digestion with trypsin, consisting of residues 644–896 of the holoenzyme, retained the bound cobalamin. A related fragment extending from residue 651 to residue 896 was generated in crystallization experiments [28]. The 28 kDa fragment stabilized bound cob(II)alamin against oxidation and protected the bound methylcobalamin against photolysis, properties that distinguish enzyme-bound from free cobalamin derivatives. However, the fragment was unable to catalyze demethylation of the methylcobalamin cofactor by homocysteine or methylation of cob(I)alamin by CH_3-H_4 folate [12•].

The 98 kDa fragment, extending from residues 1 to 896 and isolated from tryptic digests of holoenzyme containing methylcobalamin, is initially fully active in catalyzing methyl transfer from CH_3-H_4 folate to homocysteine [12•]. Thus this fragment must retain the residues necessary for binding both homocysteine and CH_3H_4 folate,

as well as residues necessary for catalysis of methyl transfer to and from the enzyme-bound cobalamin. However, the enzyme activity gradually diminishes during turnover and the cob(II)alamin enzyme can no longer be reactivated by reductive methylation with AdoMet. These observations suggest that the carboxy-terminal fragment is essential for reductive activation.

The carboxy-terminal 37 kDa unit carries the binding site for the activator, AdoMet. After intact protein has been irradiated with ultraviolet light in the presence of tritiated AdoMet, the label is associated with this fragment. The isolated fragment can also be labeled with AdoMet [12•]. However, the mechanism by which methyl transfer from AdoMet is coupled to reduction of cob(II)alamin (Fig. 1) remains to be elucidated. Several other enzymes are also activated by reactions involving flavodoxin and AdoMet: pyruvate formate–lyase [29,30], anaerobic ribonucleotide reductase [31,32•] and, possibly, biotin synthase [33,34•]. Activation of pyruvate formate-lyase results in the formation of an organic radical, localized to the α-carbon of a specific glycine residue [35•], and a glycine radical is also thought to be formed in the activation of anaerobic ribonucleotide reductase [36•,37•]. One-electron reduction of AdoMet and homolytic cleavage of the carbon–sulfur bond yields a 5′-deoxyadenosyl radical which, in turn, can generate the protein radical by hydrogen abstraction [35•]. In the case of methionine synthase, no evidence for a protein radical has been obtained, but one-electron reduction of AdoMet to form *S*-adenosylhomocysteine (AdoHcy) and a methyl radical is a possible pathway leading to formation of methylcobalamin.

Experiments to elucidate the mode of inactivation of cobalamin-dependent methionine synthase by the anaesthetic gas nitrous oxide provide evidence for close approach of the carboxy-terminal domain and the cobalamin cofactor [38•,39•]. In an electrochemical cell under nitrous oxide (N_2O) the enzyme catalyzes the one electron reduction of this gas according to equation 3 [38•]:

$$EH^+\cdot\text{cob(I)alamin} + N_2O \rightarrow E\cdot\text{cob(II)alamin} + N_2 + OH\cdot \quad (3)$$

The hydroxyl radical is presumably generated from the oxygen of nitrous oxide as this gas reacts with cobalamin bound to the 28 kDa fragment. This radical is detected because its predominant reaction is abstraction of a hydrogen atom from Val1177 in the carboxy-terminal portion of the protein, to form a valine radical that condenses with the reduced triquat dye used to mediate electron transfer to the enzyme [39•]. Since the hydroxyl radical is expected to have an extremely short lifetime, it is likely that Val1177, 50 residues from the extreme carboxyl terminus of the protein, lies close in space to the site where nitrous oxide is reduced by cob(I)alamin.

Recent sequence determinations have helped to define conserved segments of methionine synthase. Two open reading frames with homologies to the 98 kDa amino-terminal domain and the 37 kDa carboxy-terminal domain of MetH have been identified in *Mycobacterium leprae* [40]. Taken together, the two *M. leprae* sequences show 27% identity with the full-length MetH sequence of *E. coli*. As shown by the alignment in Fig. 2, the similarity of these two methionine synthase sequences is most evident in the 28 kDa cobalamin-binding portion of the *E. coli* enzyme, but other strongly conserved regions, including sequences upstream from the cobalamin-binding region, are apparent from the alignment. The roles of the amino-terminal sequences of methionine synthase remain to be determined. At present there is no direct evidence that identifies the substrate-binding sites in methionine synthase. However, alignment of MetH with the sequence for a methyl transferase from *Clostridium thermoaceticum* [41•] points to a region that may include determinants for the binding of CH_3-H_4 folate (Fig. 2). The transferase catalyzes methyl transfer from CH_3-H_4 folate to the cobalt center of a corrinoid/iron–sulfur protein, and functions in the reductive acetyl-CoA pathway for CO_2 fixation in *C. thermoaceticum*. Thus the reaction catalyzed by the methyl transferase is equivalent to one of the half reactions of methionine synthase (equation 2). The first 200 residues of the deduced methyl transferase sequence align immediately upstream from the cobalamin-binding region of methionine synthase, between residues 356 and 630, suggesting that this portion of methionine synthase includes the binding site for CH_3-H_4 folate and residues necessary to activate this substrate for removal of the methyl group. The notion that methionine synthase from *E. coli* is a modular structure is reinforced by the homology with this methyl transferase. Presumably, the major determinants for binding homocysteine and methionine, neither of which interacts with *C. thermoaceticum* methyl transferase, lie elsewhere in the methionine synthase sequence, perhaps in the amino-terminal 350 residues.

The crystal structure of the methylcobalamin-binding domains of methionine synthase

The X-ray structure of a 27 kDa cobalamin-binding region of methionine synthase has been determined at a resolution of 3.0 Å [42••]. This analysis revealed a number of cobalamin–protein interactions that are likely to be characteristic of other cobalamin-dependent proteins.

The fragment that binds methylcobalamin folds into two domains, with the corrin macrocycle embedded in the domain interface (Fig. 3). On binding to methionine synthase, methylcobalamin undergoes a dramatic rearrangement in which the dimethylbenzimidazole nucleotide swings away from the cobalt to open the lower face of the corrin to coordination by a histidine residue from the protein. The 'nucleotide tail' penetrates into the core of the carboxy-terminal α/β domain. Thus the recognition of methylcobalamin by the protein involves three sets of protein–cofactor interactions: contacts with the upper face of the corrin, where methylation and demethylation of the prosthetic

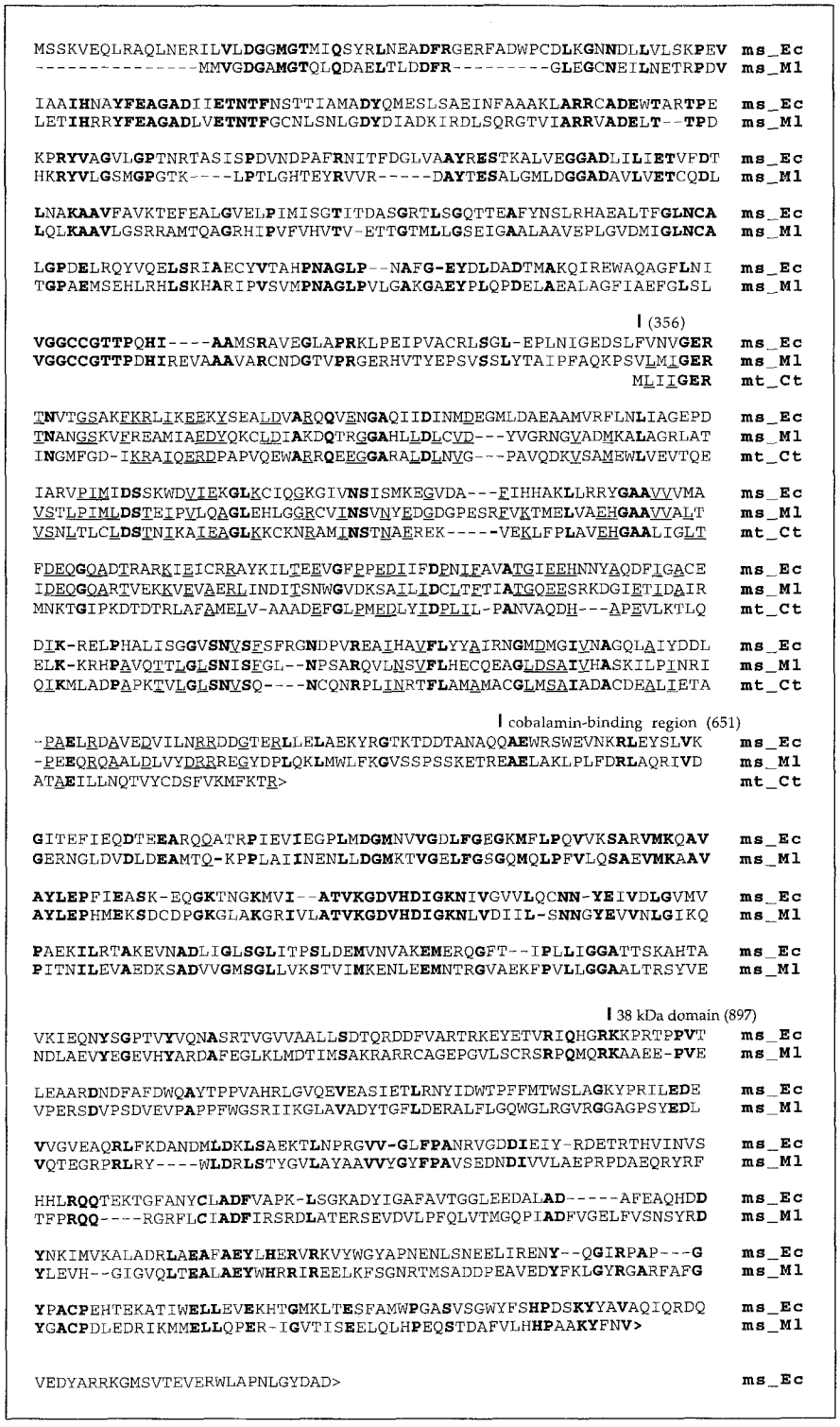

Fig. 2. Alignment of the deduced amino acid sequence of the *metH* gene from *Escherichia coli* [27•] (ms-Ec) with deduced sequences from two open reading frames from *Mycobacterium leprae* [40] (ms-Ml) that were identified by homology as coding for the 98 and 37 kDa domains of a cobalamin-dependent methionine synthase. Also shown is the alignment of the deduced sequence for a methyltetrahydrofolate–corrinoid/iron–sulfur protein methyl transferase from *Clostridium thermoaceticum* [41•] (mt-Ct). Pairwise identities are underlined in the region that includes the sequence of the methyltransferase and residues conserved in all sequences are shown in bold. Alignments were initially performed with MacVector programs, and were then modified to accord with the structure.

group take place; contacts with the dimethylbenzimidazole side chain, which help to anchor the cofactor to the protein; and contacts with the lower face, which allow the protein to modulate the reactivity of the cobalt ion.

The amino-terminal domain of the fragment, a four-helix bundle [43], provides interactions with the upper face of methylcobalamin. In methionine synthase, the upper face contacts are of two types. Hydrophobic side chains such as Val704, Phe708, Leu715 and Leu718 contact the methyl group bonded to cobalt as well as other non-polar substituents of the corrin, while backbone carbonyls hydrogen-bond to the characteristic acetamide side chains of the corrin (Fig. 4). It is expected that this region will differ in enzymes that use adenosyl-

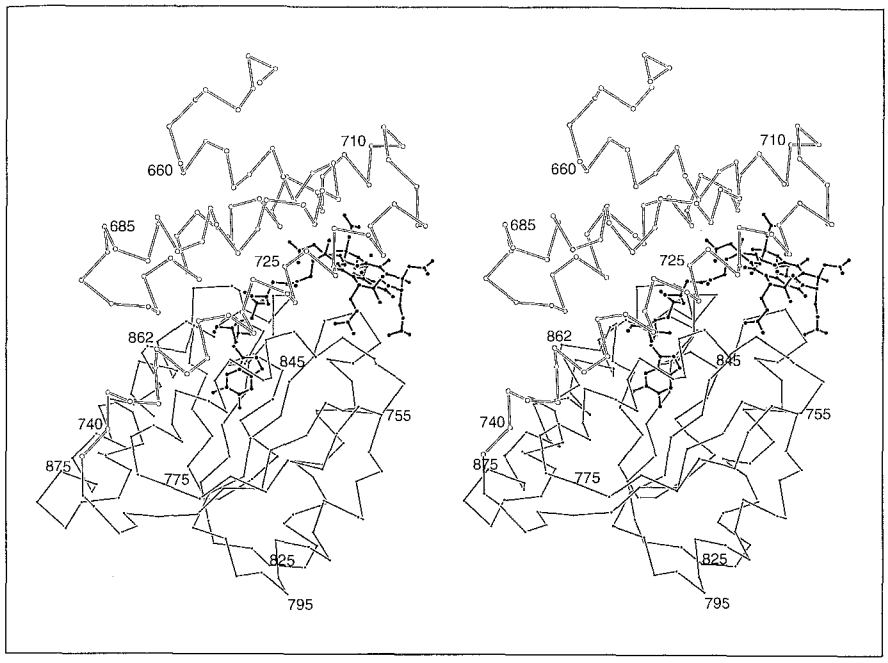

Fig. 3. A stereoview of the fold of the cobalamin-binding domains of methionine synthase with methylcobalamin bound. The fold and topology of the amino-terminal helical domain (open bonds) resembles the arrangement of the E, F, G and H helices of the globins, but the corrin is bound on the outside of the helix bundle rather than in its core. The carboxy-terminal α/β domain (thin filled bonds) belongs to the general family of nucleotide-binding folds but, as shown in Fig. 5, has evolved features that specifically recognize the lower face and side chain of the cobalamin prosthetic group.

cobalamin and, indeed, no sequence similarities between the methyl- and adenosylcobalamin-dependent proteins have been detected in this vicinity.

In order to accommodate the unique structure of cobalamin, the carboxy-terminal α/β domain varies significantly from the canonical nucleotide-binding fold (Rossmann fold). Central elements of this domain surround the dimethylbenzimidazole substituent of the corrin, forming an elongated pocket that accommodates the nucleotide tail (Figs 3 and 4). The third and fourth strands of the parallel β-sheet line one side of the pocket, which is completed by residues from helices IIα1 and IIα5. The buried surface area of the side chain carrying the dimethylbenzimidazole nucleotide accounts for approximately one-half of the total buried area of bound methylcobalamin, suggesting that the interactions of the displaced side chain are important contributors to the overall binding of the cofactor. Comparisons with the nucleotide-binding domain of lactate dehydrogenase (Fig. 5) reveal that helices are not pried away from the sheet to make space for the dimethylbenzimidazole nucleotide. The dimethylbenzimidazole ring system is accommodated partly by the substitution of glycines for the larger side chains of lactate dehydrogenase. In methionine synthase, Gly762, at the start of helix IIα1, Gly802 from strand IIβ3 and Gly833 from

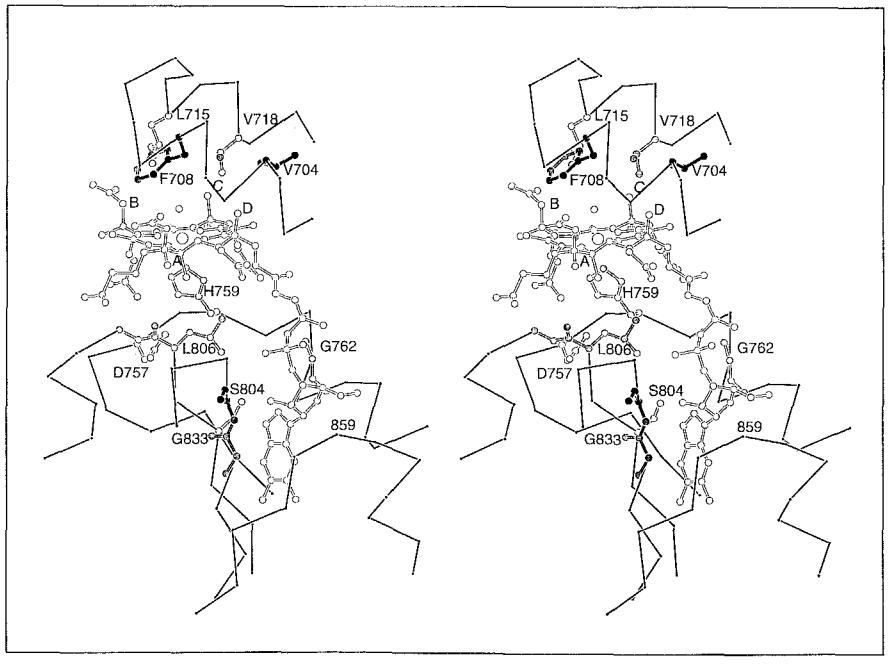

Fig. 4. A close-up view of the methylcobalamin-binding site, rotated 180° from the vantage point of Fig. 3. The conserved 'fingerprint' residues from the α/β domain, drawn in full-atom mode, are below the corrin ring. Hydrophobic residues from the helical domain that contact the upper face of the corrin are also displayed in detail.

strand IIβ4 replace the valines found in corresponding sites in lactate dehydrogenase. Truncation of helix IIα5 at its amino terminus (near residue 860) opens a site for the ribose-3′-phosphate moiety of cobalamin. Figure 5 shows the relationship between the binding sites for the dimethylbenzimidazole nucleotide of cobalamin and the nicotinamide riboside of oxidized nicotinamide adenine dinucleotide (NAD^+). The conformations of the loops at the carboxy-termini of the β-sheet are another distinquishing feature of the α/β domain of methionine synthase. The connecting loop between strand IIβ1 and helix IIα1 contributes the His759 ligand to the cobalt instead of binding a nucleotide phosphate. The classic binding site for the adenine nucleotide of pyridine nucleotide-dependent dehydrogenases is obliterated by this loop (Fig. 5). A second loop connecting strand IIβ3 with helix IIα3 is extended at residue 805 and provides hydrophobic contacts with the corrin and polar interactions with propionamide side chains.

A network of hydrogen-bonded residues beneath the corrin may be important in the catalytic reactions of methionine synthase

A hydrogen-bonded network connects the cobalt and the lower axial ligand, His759, to Ser810 at the surface of the cobalamin-binding fragment and provides a possible pathway for transfer of protons to and from residues near the cobalamin. The δ-nitrogen of the His759 ligand is positioned to form a hydrogen bond to one oxygen of Asp757 (Fig. 4), and Ser810 is oriented to a hydrogen-bond to the other aspartate oxygen. During turnover, methionine synthase must alternately stabilize

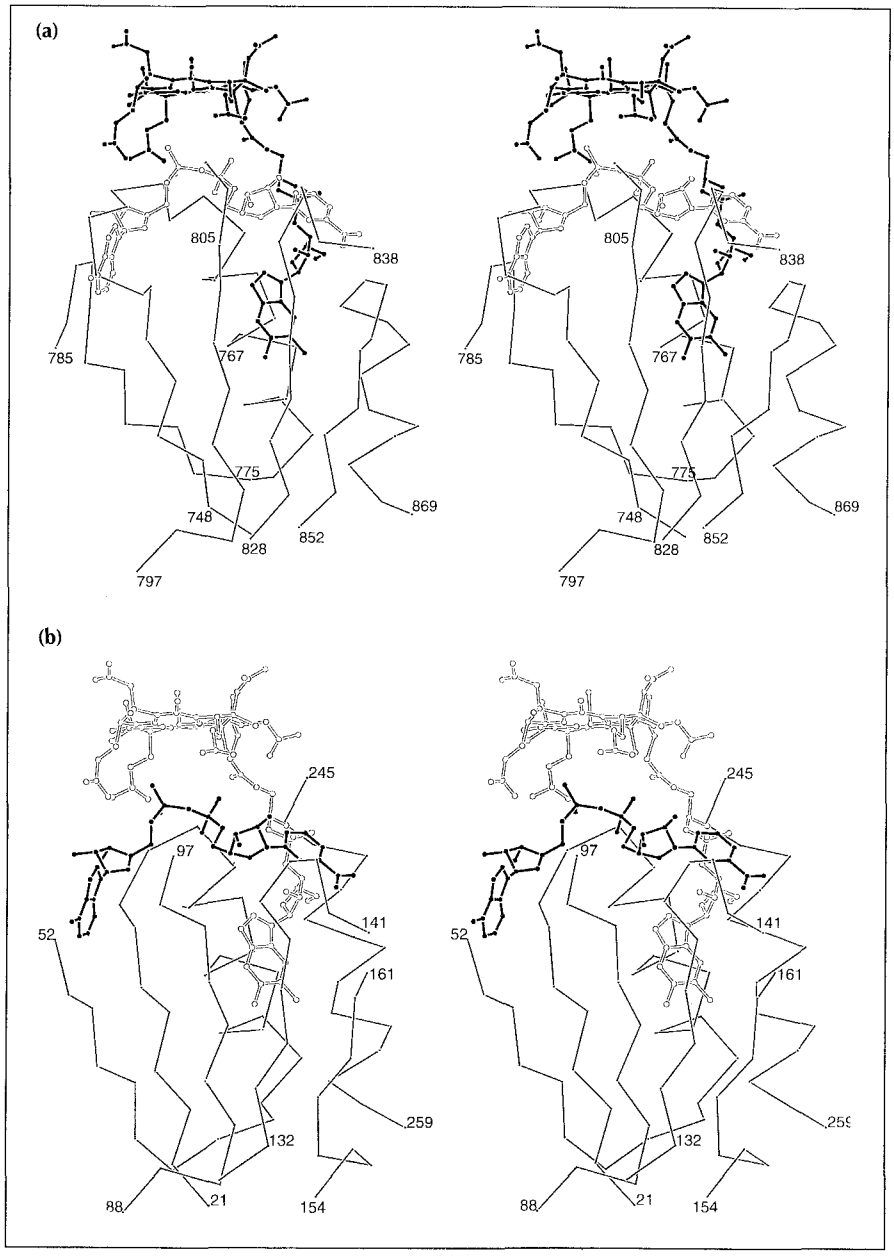

Fig. 5. Comparisons of the folds and cofactor binding of the α/β domains of methionine synthase and lactate dehydrogenase [61]. The structures were aligned by matching the backbones of the four central sheet strands. **(a)** Cobalamin (closed bonds), β-sheet and other backbone features of methionine synthase, with the oxidized nicotinamide adenine dinucleotide (NAD^+) of lactate dehydrogenase superimposed. **(b)** Corresponding β-sheet and bound NAD^+ of lactate dehydrogenase, with the methylcobalamin of methionine synthase (open bonds) superimposed. Although the corrin macrocycle adjoins the amino-termini of the central sheet strands in methionine synthase, it is significantly displaced from the α/β domain, relative to the bound pyridine nucleotide. The adenine nucleotide binding site of lactate dehydrogenase has been replaced by residues 753–760 and 780–783 of methionine synthase. Like the nicotinamide nucleotide of lactate dehydrogenase, the dimethylbenzimidazole side chain of the corrin lies on the side of the sheet away from the viewer. However, the sizes and shapes of the binding sites are affected by residue changes (see text), by loops at the ends of strands β1 and β4, and by the amino terminus of helix IIα5, all of which differ between the two structures. It is clear from these superpositions that the pocket for dimethylbenzimidazole is not equivalent to the classical binding sites for the adenine moieties of pyridine- or flavin-dinucleotides.

methylcobalamin and cob(I)alamin forms of the prosthetic group (Fig. 1). In model cobalamins, these forms are six- and four-coordinate, respectively. Formation of cob(I)alamin in methionine synthase is thus assumed to involve dissociation of the His759 ligand. Protonation and deprotonation of the His759–Asp757 pair, perhaps mediated by Ser810, may play an important role in the conversion between methylcobalamin and cob(I)alamin species [42••]. We have suggested that the His-Asp pair is deprotonated in the methylcobalamin form of the enzyme, with the histidine partially ionized to the imidazolate form. Protonation of the histidine–aspartate pair accompanying demethylation would favor formation of the cob(I)alamin form of the enzyme. While there is no direct evidence yet for this proposed change in protonation state on going from methylcobalamin to cob(I)alamin, there is direct evidence for the uptake of a proton when the enzyme-bound cofactor is reduced from cob(II)alamin to cob(I)alamin [38•].

In cytochrome c peroxidase, hydrogen-bonded histidine and aspartate residues are located below the heme iron in an arrangement similar to that seen in methionine synthase In this system as well, reduction of the iron is associated with proton uptake and the redox properties of the heme iron are very sensitive to the precise geometry of the hydrogen-bonding network [44••].

Evidence for structural conservation of the cobalamin-binding α/β domain in adenosylcobalamin-dependent enzymes

Information from the methionine synthase structure and sequence alignments with a subset of six adenosylcobalamin-dependent enzymes (Fig. 6) have been combined to define fingerprint sequences that indicate cobalamin binding. These motifs include many of the conserved residues originally noted by Marsh and co-workers [45]. Although the number of identical residues (bold, Fig. 6) in the alignment is small, many of these invariant residues are in direct contact with cobalamin atoms (Fig. 4) and their conservation can be rationalized from the structure. The paucity of conserved residues may reflect the observation that most of the contacts between the cobalamin side chains and the protein involve either hydrophobic interactions or hydrogen-bonding to backbone atoms rather than to polar side chains of the protein.

The sequences $DxH^{759}xxG$ (where x is any amino acid), SxL^{806} and G^{833}–G^{834} seem to be diagnostic for cobalamin binding. The conserved motif $DxH^{759}xxG$ forms a loop that passes under the corrin macrocycle, and includes His759, the lower axial ligand to the cobalt [42••]. As part of the network for protonation described in the previous section, Asp757 is hydrogen-bonded to the δ-nitrogen of His759, while the side chain of Leu806 contacts the lower face of the corrin and packs against the imidazole ring of His759 (Fig. 4). The remaining four residues of the fingerprint sequences, Gly762, Ser804 and Gly833–Gly834, interact with the nucleotide tail of the cobalamin (Fig. 4). Gly 762 is located at the beginning of helix IIα1, where a side chain would overlap with the phosphate and sugar of the dimethylbenzimidazole nucleotide. The Gly833–Gly834 doublet also appears to be conserved for steric reasons. Side chains in these positions at the carboxyl terminus of IIβ4 would block the channel into which dimethylbenzimidazole is inserted. The conserved Ser804 is in position to hydrogen-bond to the N^3 of dimethylbenzimidazole in the nucleotide tail.

The conservation of hydrophobic and hydrophilic patches, and the conservation of residues involved in unusual structural features such as a β bulge at the carboxy-terminal end of strand IIβ2, predicts that folds closely related to the α/β domain of methionine synthase will be found in methylmalonyl CoA mutase and glutamate mutase. The alignments of hydrophobic residues corresponding to the residues in strands IIβ1, IIβ3, and IIβ4, which constitute the core of the Rossmann-like domain, are particularly impressive (Fig. 6) The sequence alignments strongly suggest that the fundamental features of the interactions at the lower face of cobalamin, particularly the replacement of dimethylbenzimidazole by a histidine ligand from the protein, will be retained in these adenosylcobalamin-dependent enzymes. In glutamate mutase, the homologous domain is an independently folded subunit of the enzyme [45,46,47•], while in methylmalonyl CoA mutase and methionine synthase this domain is embedded in a larger polypeptide.

The deduced amino acid sequences of some other adenosylcobalamin-dependent enzymes cannot readily be aligned with methionine synthase. However, the conserved sequence DxH from the cobalamin-dependent ribonucleoside triphosphate reductase from *Lactobacillus leichmanii* [48•] and from mycobacteriophage L5 [49] may be a fingerprint sequence surrounding a histidyl ligand to adenosylcobalamin, and the large subunits of the ethanolamine ammonia-lyases from *Salmonella typhimurium* [50] and *Rhodococcus* sp. NI86/21 [51] contain the sequences GlEdHfcG and GlEdHfmG, respectively. Blakley *et al.* [52] reconstituted *L. leichmanii* ribonucleoside triphosphate reductase with a cobalamin analog containing an adenine nucleotide that assumes a 'base-off' conformation in solution. Electron paramagnetic resonance spectroscopy of the protein reconstituted with the cob(II)alamin analog showed the characteristic nitrogen superhyperfine interactions that indicate coordination of a nitrogen in the lower axial (α) position of the cobalamin. As pointed out by the authors at that time, one interpretation of this finding is that a nitrogenous ligand from the protein becomes coordinated to the cobalt as the base-off analog binds in the active site.

Differential reactivity of protein-bound cobalamins

Despite the evidence of structural similarity between methionine synthases and adenosylcobalamin-dependent mutases, these two classes of enzymes catalyze heterolytic

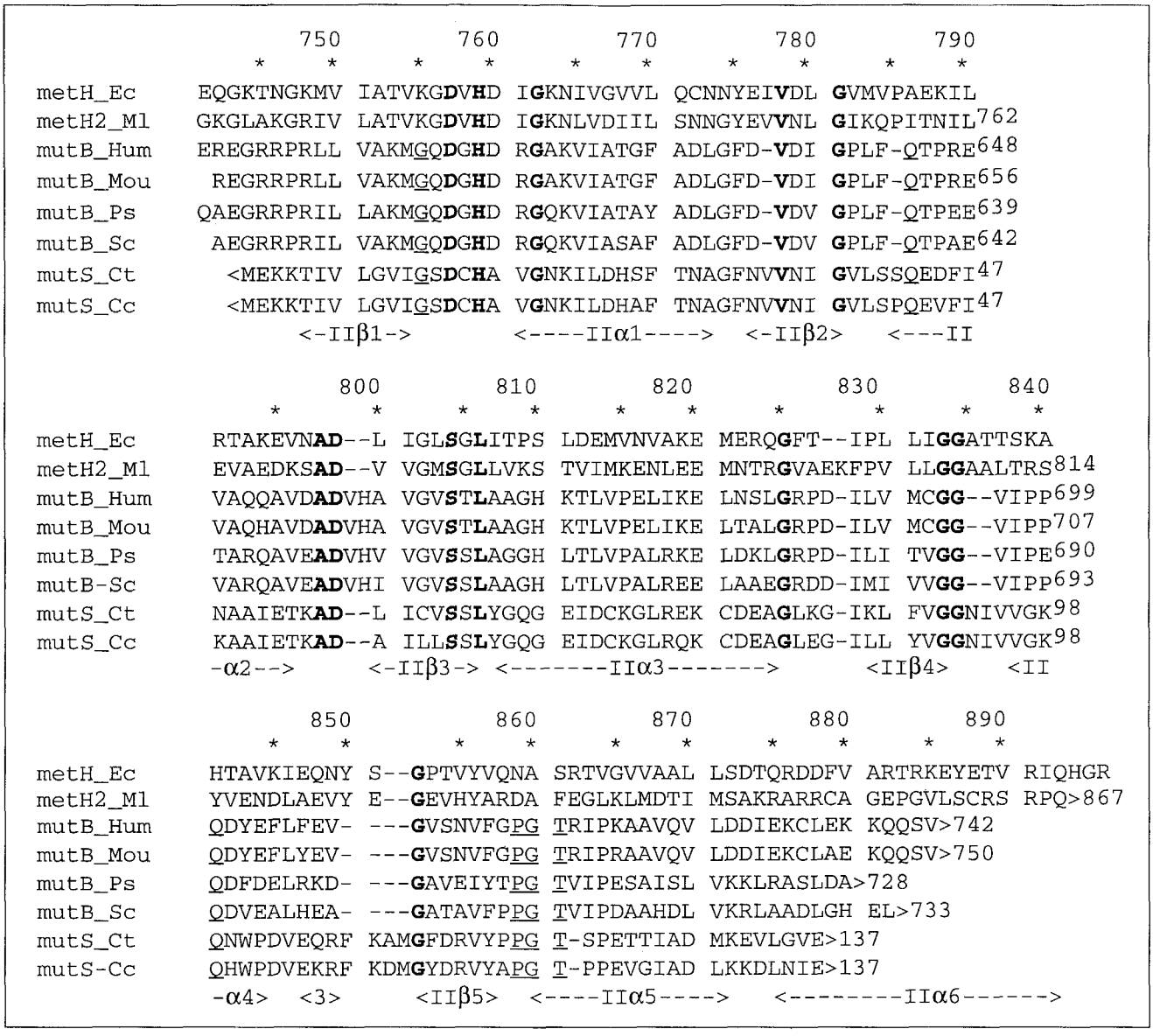

Fig. 6. Alignment of deduced amino acid sequences of a subset of adenosylcobalamin-dependent mutases with homologies to the α/β (Rossmann) domain of methionine synthase. Marsh and his co-workers [45] first reported some of the homologies indicated here. The sequences shown are: residues 740–896 of the deduced amino acid sequence of methionine synthase from *Escherichia coli*, designated metH-Ec (GenBank Accession #J04975 [27•]); residues 712–867 of the deduced amino acid sequence of open reading frame *metH2* from *Mycobacterium leprae* [40], designated metH2-M1; residues 599–742 of the deduced amino acid sequence obtained from the human cDNA for methylmalonyl-coenzyme A (CoA) mutase [62], designated mutB-hum; residues 610–750 of the deduced amino acid sequence of the mouse methylmalonyl-CoA mutase (GenBank accession #X57941 [63]), designated mutB-mou; residues 593–728 of the deduced amino acid sequence of the methylmalonyl-CoA mutase MutB subunit from *Propionibacterium shermanii* (GenBank accession #X14965 [64]), designated mutB-Ps; residues 596–733 of the deduced amino acid sequence of the methylmalonyl-CoA mutase MutB subunit from *Streptomyces cinnamonensis* [65]; residues 1–137 comprising the small MutS subunit of glutamate mutase from *Clostridium tetanomorphum* [45], designated mutS-Ct; and residues 1–137 comprising the deduced amino acid sequence of the *glmS* gene encoding the small subunit of glutamate mutase from *Clostridium cochlearium* [46], designated mutS-Cc. Residues shown in bold are conserved in all the aligned sequences; residues that appear to be conserved in adenosylcobalamin-dependent enzymes but not in the methylcobalamin-dependent methyl transferases are underlined. Varations between adenosyl and methyl cobalamin enzymes that could be important include the position of a glycine immediately upstream of the DxHxxG and the appearance of two conserved glutamine residues in all the mutases, corresponding to Ala785 and His841 in MetH. Ser810, a member of the hydrogen-bonded network in methionine synthase, conserved in the methionine synthase from *Mycobacterium leprae*, is absent from the mutases, which do not form cob(I)alamin during the catalytic cycle.

cleavage of the carbon–cobalt bond on the one hand, and homolytic cleavage on the other. A salient feature of several adenosylcobalamin enzymes is a striking enhancement of the rate of carbon–cobalt bond cleavage on binding of the cofactor to the enzyme–substrate complex [53]. Two basic theories have been proposed to explain the reactivity of cobalamins: a chemical theory holding that the basicity of the lower ligand is the key in modulating the reactivity of the cobalt, and a 'mechanical' theory proposing that deformation of the corrin

ring system modulates the strength of the carbon–cobalt bond.

The mechanical theory arose from X-ray analyses of a variety of corrins which displayed an 'upward' deformation of the corrin-ring plane [54,55]. Repulsive forces between the deformed ring and a bulky upper ligand could weaken the carbon-cobalt bond. The recent X-ray structure determination of a vitamin B_{12} derivative in which imidazole has been substituted for dimethylbenzimidazole in the nucleotide loop [56•] shows a decrease in the folding angle of the ring system from 18.0 °to 11.3°, suggesting that the bulky dimethylbenzimidazole is primarily responsible for deformation. For adenosylcobalamin-dependent mutases in which histidine is the lower ligand, it is hard to see how ring flexure can account for increased rates of carbon–cobalt bond cleavage.

Model studies suggest that the ligand in the lower axial position significantly influences the strength of the carbon–cobalt bond in the upper axial position and the propensity for homolytic versus heterolytic cleavage [57–60]. For those adenosylcobalamin enzymes that share the DxH motif with methionine synthase, subtle differences must underlie the different reactivities. As mutational analysis of cytochrome c peroxidase indicates, the reactivity of the metal is highly sensitive to small perturbations in hydrogen bonding of the lower ligand [44••]. Studies of mutant methionine synthases and structural and mutational studies of the mutase enzymes will be crucial for an incisive analysis of the reactivity of protein-bound cobalamins.

Conclusions

The X-ray structure of the cobalamin-binding region of methionine synthase has revealed unexpected similarities between methylcobalamin-dependent methionine synthase and the adenosylcobalamin-dependent methylmalonyl CoA and glutamate mutases. In particular, it seems very likely that these mutases will bind the cobalamin cofactor with a histidyl residue from the protein displacing the dimethylbenzimidazole nucleotide. The α/β domain, a variant of the Rossmann fold, is responsible for stabilizing the dimethylbenzimidazole nucleotide in its displaced position and appears to be structurally conserved in these enzymes. This domain shows significant local divergence from the canonical flavin- or pyridine-nucleotide binding domains. Because of its size, the corrin macrocycle does not enter the crevice that is occupied by nucleotides in the pyridine nucleotide-dependent dehydrogenases. The characteristic site that binds the AMP moiety of NAD^+ has been lost, and the region that binds the nicotinamide mononucleotide moiety has been reshaped and enlarged to admit the long dimethylbenzimidazole tail. The α/β domain also contains residues thought to be responsible for modulating the carbon–cobalt bond strength of the methyl- or adenosylcobalamin cofactor, and its detailed structure is predicted to be extremely important in determining whether cleavage of the carbon–cobalt bond is heterolytic or homolytic.

Note added in proof

Ruma Banerjee and her co-workers [66•] have recently purified methionine synthase from porcine liver to homogeneity, with retention of high activity. They have shown that the porcine enzyme is a monomer of ~155 kDa, and that it exhibits an ordered sequential kinetic mechanism similar to that observed for the prokaryotic enzyme.

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