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Methylenetetrahydrofolate reductase and methionine synthase: biochemistry and molecular biology

Abstract Methylenetetrahydrofolate reductase and cobalamin-dependent methionine synthase catalyze the penultimate and ultimate steps in the biosynthesis of methionine in prokaryotes, and are required for the regeneration of the methyl group of methionine in mammals. Defects in either of these enzymes can lead to hyperhomocysteinemia. The sequences of the human methylenetetrahydrofolate reductase and methionine synthase are now known, and show clear homology with their bacterial analogues. Mutations in both enzymes that are known to occur in humans and to be associated with hyperhomocysteinemia affect residues that are conserved in the bacterial enzymes. Structure/function studies on the bacterial proteins, summarized in this review, are therefore relevant to the function of the human enzymes; in particular studies on the effects of bacterial mutations analogous to those causing hyperhomocysteinemia in human may shed light on the defects associated with these mutations.

Key words Hyperhomocysteinemia · Polymorphism · Mutations · Cobalamin · Vitamin B_{12}

Abbreviations *AdoMet* S-adenosylmethionine · *AdoHcy* adenosylhomocysteine

Introduction

With the growing interest in the clinical sequelae associated with homocysteinemia, attention has been directed towards studies of the enzymes responsible for the generation and consumption of homocysteine. As shown in Fig. 1, methylenetetrahydrofolate reductase and methionine synthase are two such enzymes. Methylenetetrahydrofolate reductase catalyzes the reduction of methylenetetrahydrofolate to methyltetrahydrofolate. This is the only reaction generating methyltetrahydrofolate in the cell. Methionine synthase catalyzes a methyl transfer from methyltetrahydrofolate to homocysteine, generating methionine and tetrahydrofolate. In bacteria, the reaction catalyzed by methionine synthase is the terminal reaction in the de novo biosynthesis of methionine; in humans, for whom methionine is an essential amino acid, this reaction serves to regenerate the methyl group of methionine. As indicated in Fig. 1, methionine is converted to adenosylmethionine (AdoMet), which serves as a methyl donor in numerous biosynthetic reactions. The product adenosylhomocysteine (AdoHcy) is then hydrolyzed to form adenosine and homocysteine. Homocysteine can be reconverted to methionine to provide another methyl group.

Homocysteine is a metabolite at a critical branch point in 1-carbon metabolism. If the cell is replete with AdoMet, and the ratio of AdoMet/AdoHcy is high, methylenetetrahydrofolate reductase is inhibited. Under these conditions the level of methyltetrahydrofolate in the cell is low, and homocysteine is degraded by conversion to cystathionine and thence to α-ketobutyrate, ammonia, and cysteine. If the ratio of AdoMet/AdoHcy is low, signalling a need for synthesis of more AdoMet, methylenetetrahy-

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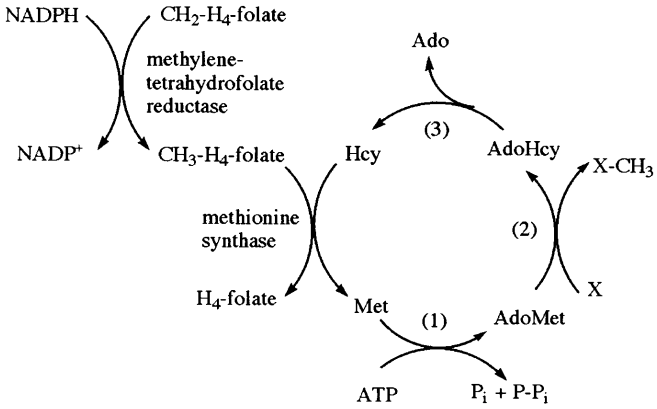

Fig. 1 Pathways involved in the production of methyltetrahydrofolate and in the regeneration of homocysteine to form methionine. Enzymes *(1)* are the methionine adenosyltransferase isozymes, enzymes *(2)* are AdoMet-dependent methyltransferases, and enzyme *(3)* is S-adenosylhomocysteine hydrolase. *CH_2-H_4folate* methylenetetrahydrofolate, *CH_3-H_4folate* methyltetrahydrofolate, *H_4folate* tetrahydrofolate, *AdoHcy* adenosylhomocysteine, *P_i* phosphate anion, *P-P_i* pyrophosphate anion

drofolate reductase inhibition is relieved, and methyltetrahydrofolate is produced to support the reaction of methionine synthase. Studies by Kutzbach and Stokstad [24] demonstrated that methylenetetrahydrofolate reductase acitvity is allosterically regulated by the AdoMet/AdoHcy ratio, with AdoMet serving as an inhibitor, and AdoHcy competing with AdoMet for binding to the reductase but not acting as an inhibitor.

Defects in either methylenetetrahydrofolate reductase [21, 22, 31] or methionine synthase [35, 41] can lead to hyperhomocysteinemia, as can deficiencies in β-cystathionase activity [2]. Severe defects, which lead to greatly elevated blood homocysteine levels, were the first to be identified, but recently we have realized that defects that lead to mild elevations in blood homocysteine levels are potential long-term risk factors [13, 37, 43]. Such mild defects may be associated with polymorphisms.

During the past 3 years, the nucleotide sequences for the human cDNAs specifying methylenetetrahydrofolate reductase [16] and methionine synthase [3, 25, 26] have been published. The availability of these sequences permits the identification of mutations that lead to impaired function of these enzymes, and hence to homocysteinemia. We are thus increasingly able to identify humans at risk for homocysteinemia and its sequelae. Extensive structural and functional studies of either the mammalian enzymes or their prokaryotic analogues have been performed, and thus studies can assist in understanding the defects associated with specific mutations, and can suggest strategies to ameliorate the symptoms caused by these mutations.

Methylenetetrahydrofolate reductase

Most of our knowledge about the structure and function of human methylenetetrahydrofolate reductase derives from studies of the closely related porcine enzyme. This enzyme was initially characterized by Kutzbach and Stokstad, and has subsequently been extensively studied in our laboratory. Kutzbach and Stokstad [24] partially purified the enzyme from porcine liver, and showed that it was allosterically regulated by AdoMet. The enzyme was subsequently purified to homogeneity [5], and shown to contain one equivalent of non-covalently bound FAD per enzyme subunit. The enzyme is a dimer of identical 77 kDa subunits. Tryptic proteolysis of the native enzyme was shown to cleave each subunit into two fragments, an N-terminal 40 kDa fragment and a C-terminal 37 kDa fragment [30]. Tryptic cleavage results in loss of allosteric regulation of enzyme activity by AdoMet, but has no effect on the catalytic activity of the enzyme, suggesting that the protein may consist of separate catalytic and regulatory regions [30]. AdoMet was subsequently shown to bind to the C-terminal 37 kDa fragment, implicating this fragment as the regulatory region [38].

The deduced amino acid sequence of the human enzyme provided further insight into the functional organization of methylenetetrahydrofolate reductase. The N-terminal region of the human protein showed extensive similarity with smaller proteins from enteric bacteria that catalyze the same reaction, namely the NAD(P)H-dependent reduction of methylenetetrahydrofolate. Since the activity of these enzymes is not allosterically regulated by AdoMet, there was a strong inference that the N-terminal region of the human enzyme is the catalytic region, and contains determinants for binding of FAD, NADPH, and methylenetetrahydrofolate. The C-terminal region of the human enzyme shows sequence similarities with the enzymes from yeast and from the roundworm *Caenorhabditis elegans* but this region is lacking in the bacterial methylenetetrahydrofolate reductases. The human enzyme contains an extremely hydrophilic region, Lys-Arg-Arg-Glu-Glu-Asp, that bridges the catalytic and regulatory regions; cleavage between Lys and Arg residues in this region would divide the protein into 40 and 34 kDa fragments. The sequence of a peptide in the porcine enzyme that is labeled by irradiation of methylenetetrahydrofolate reductase in the presence of [^{3}H-methyl] AdoMet has been determined; a sequence similar to this peptide is located immediately downstream of the tryptic cleavage site [16].

Methylenetetrahydrofolate reductase from *Escherichia coli* had only previously been characterized in relatively impure preparations [23], and we have developed a method to purify this enzyme to homogeneity from an overexpressing strain. The purified enzyme is a flavoprotein, and contains non-covalently bound FAD as its cofactor. The enzyme-bound flavin is reduced by NADH, and much more slowly by NADPH, and can in turn reduce methylenetetrahydrofolate to methyltetrahydrofolate.

Rozen and her collaborators [13, 16, 17] have conducted an extensive search for mutations in the human methylenetetrahydrofolate reductase gene, concentrating especially on mutations in the N-terminal catalytic region. Several mutations associated with severe deficiency in pa-

tients have been identified, two such point mutations are Arg157Gln, and Thr227Met. A polymorphism, Ala222Val, has been shown to be present in high frequency in humans; in a population of French Canadians Ala/Val heterozygotes are present at 51% frequency, and Val/Val homozymgotes at 12% frequency [13]. Humans who are homozygous for the polymorphism have reduced specific activity of methylenetetrahydrofolate reductase in fibroblast extracts, and demonstrate increased susceptibility to heat inactivation of enzyme activity (as assessed by measuring reductase activity after heating for 5 min at 46° C and comparing with the activity of controls) [13]. The Val/Val genotype has subsequently been shown to be associated with increased risk for neural tube defects [40, 42], and possibly for the develoment of cardiovascular disease [11, 28], although not all studies have found significant correlations [6]. Each of the mutated residues, Arg157, Thr227, and Ala222, is conserved in the bacterial, yeast, and roundworm sequences of methylenetetrahydrofolate reductase.

Because the human methylenetetrahydrofolate reductase has not yet successfully been overexpressed and purified, we have constructed a homologous mutation to Ala222Val in the methylenetetrahydrofolate reductase from *E. coli*, Ala177Val. This mutation leads to diminished expression of methylenetetrahydrofolate reductase in an overexpressing strain, and the enzyme activity is rapidly lost during purification. We have successfully purified the mutant bacterial enzyme to homogeneity by introducing a histidine tag at the C-terminus of the protein and purifying the enzyme on nickel Sepharose. Our present studies suggest that the Ala177Val bacterial enzyme is indeed thermolabile, and that it readily loses its flavin cofactor on dilution of the protein.

The reduced specific activity of methylenetetrahydrofolate reductase in humans with the thermolabile mutation [11], and the resultant elevation in homocysteine in patients with low folate status [19], may similarly reflect the presence of inactive apo-enzyme in the cells of these individuals.

Methionine synthase

Cobalamin-dependent methionine synthase from *E. coli* was initially characterized in the laboratories of Wood, Weissbach, and Huennekens (reviewed in [29]). These studies established the participation of the cobalamin (B_{12}) cofactor in the methyl transfer from methyltetrahydrofolate to homocysteine, and defined the requirements for catalytic turnover. As shown in Fig. 2, the cofactor cycles in catalysis between the methylcobalamin form and the cob(I)alamin form. Cob(I)alamin is a strong reductant, and is occasionally oxidized during catalytic turnover in the presence of oxygen to produce the inactive cob(II)alamin form of the enzyme. Return of this form of the enzyme to the catalytic cycle requires a reductive methylation, in which the methyl group is provided by AdoMet [27]; in *E. coli*, reduced flavodoxin serves as the electron donor [14].

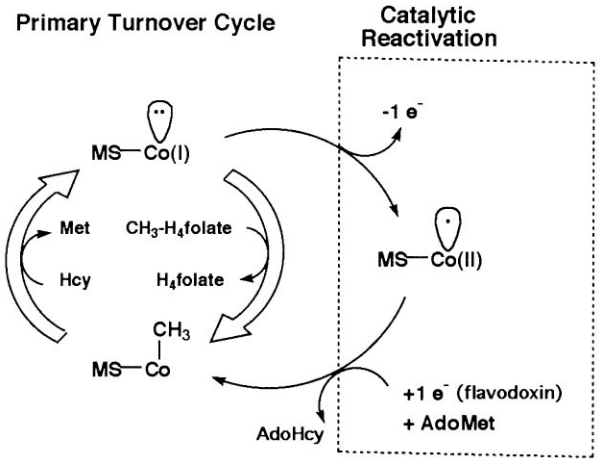

Fig. 2 Chemistry of the methionine synthase *(MS)* reaction. In normal catalysis the B_{12} prosthetic group cycles a between cob(I)alamin (CO(I)) and methylcobalamin (Co-CH_3). Homocysteine demethylates methylcobalamin to generate methionine and cob(I)alamin and the latter is remethylated by methyltetrahydrofolate (CH_3-H_4folate) with formation of tetrahydrofolate. Occasionally, the cob(I)alamin form of the enzyme becomes oxidized to the inactive cob(II)alamin form of the enzyme. Return of this form of the enzyme to the catalytic cycle requires a reductive methylation. In *E. coli*, the electron is supplied by reduced flavodoxin; the electron donor in mammals has not yet been identified. AdoMet supplies the methyl group for reductive activation

Thus catalytic turnover in the presence of oxygen requires homocysteine and methyltetrahydrofolate, AdoMet and a reducing system.

The *E. coli* methionine synthase was first cloned, overexpressed and sequenced in 1989–1990 [1, 32, 33]. As noted above, the sequence of the human methionine synthase has been simultaneously determined in three laboratories this year [3, 25, 26]. The human enzyme shows 58% identity with methionine synthase from *E. coli* [3]. These two sequences, as well as sequences from the roundworm *Caenorhabditis elegans* [39], and the prokaryotes *Hemophilus influenzae* [12], *Mycobacterium leprae* [36], and *Synechocystis sp.* strain PCC6803 [20], are aligned in Fig. 3. Given the high degree of conservation of amino acid residues throughout the proteins, the enzymes from these organisms are likely to have very similar properties and structures. Thus the large body of information available for the *E. coli* enzyme is likely to be relevant to the human enzyme.

The porcine methionine synthase has been purified to homogeneity [4] and shown to be similar in size and properties to the enzyme from *E. coli*. The one respect in which the mammalian enzymes clearly differ from the bacterial proteins is in the nature of the biological reducing system. Mammals lack flavodoxin, necessary for reductive reactivation in *E. coli*; the proteins responsible for reductive activation in mammals have not yet been identified.

Our recent studies on the bacterial enzyme have shown it to be a modular protein, consisting of four regions that are designated in Fig. 3. The N-terminal module (residues 1–353 in the *E. coli* sequence) is responsible for binding

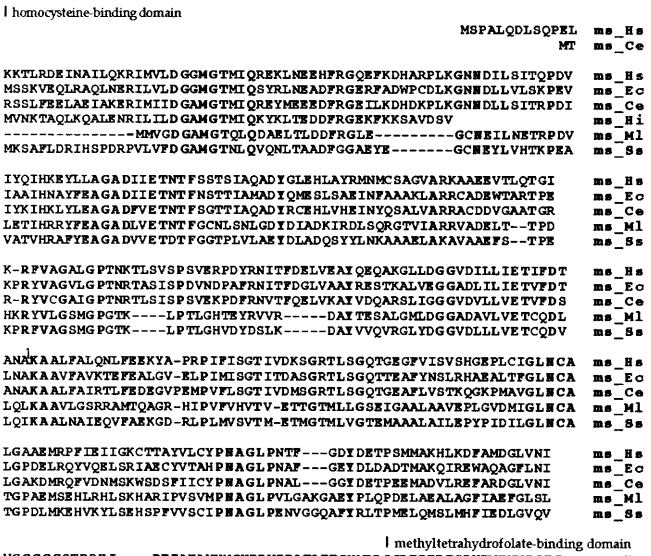

Fig. 3 Aligment of the methionine synthase sequence from Homo sapiens [3, 25, 26] with the sequence from the *E. coli* metH gene [1, 10, 33], and homologous sequences from the roundworm *Caenorhabditis elegans* [39], and the prokaryotes *Hemophilus influenzae* [12]. *Mycobacterium leprae* [36], and *Synechocystis sp.* strain PCC6803 [20]. Identical residues are shown in bold. The *Hemophilus* sequence appears to lack most of the N-terminal portion of the sequence of the other genes, perhaps because it was obtained by shotgun cloning of the entire genome

and activation of homocysteine (Goulding and Matthews, unpublished data) and shows sequence similarity with human betaine-homocysteine methyltransferase [15]. The next module, comprising residues 354–649, is involved in the binding and activation of methyltetrahydrofolate. This module shows sequence similarities with a methyltransferase from *Clostridium thermoaceticum* that catalyzes methyl transfer from methyltetrahydrofolate to a separate corrinoid iron/sulfur protein [34]. The third module of the bacterial enzyme, comprising residues 650–896, contains the determinants for binding the cobalamin cofactor [1, 8, 34], and presents the cofactor to the other three modules. A high resolution X-ray structure of this module has been determined [8]. The fourth and last module, containing residues 897–1227, binds AdoMet and is required for reductive activation of methionine synthase [9]. An X-ray structure of this module, with AdoMet bound, has recently been determined [7].

Thus, as mutations and polymorphisms of human methionine synthase are identified, we may be able to guess at their probable phenotypes from their location in the sequence. One such mutation, Pro1137Leu, is associated with severe disease in humans [18]. This mutation is located in the activation module of methionine synthase, adjacent the bound AdoMet. This mutation has a very interesting phenotype; it is active when assayed using a chemical reducing system, but shows very low activity when assayed with the biological reducing system [18]. Thus this mutation in the activation domain of methionine synthase appears to have altered the interaction of human methionine synthase with its biological reducing agent.

Analysis of other mutations and polymorphisms may not only tell us whether methionine synthase mutations are independent risk factors for neural-tube defects and/or cardiovascular disease, but may also tell us much about the role of the methionine synthase protein in catalyzing methyl transfer.

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