Defects in Auxiliary Redox Proteins Lead to Functional Methionine Synthase Deficiency*

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Methionine synthase catalyzes a methyl transfer reaction from methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate and is dependent on methylcobalamin, a derivative of vitamin B12, for activity. Due to the lability of the intermediate, cob(I)alamin, the activity of methionine synthase is additionally dependent on a redox activation system. In bacteria, two flavoproteins, NADPH-flavodoxin reductase and flavodoxin, shuttle electrons from NADPH to methionine synthase. Their mammalian counterparts are unknown, and a putative intrinsic thiol oxidase activity of the mammalian methionine synthase has been proposed to be involved. We demonstrate that the mammalian methionine synthase can be activated in an NADPH-dependent reaction and requires a minimum of two redox proteins. This model is consistent with our results from biochemical complementation studies between cbIG and cbIE cell lines and mutation detection analysis in cbIG cell lines. These demonstrate that the cbIG cell line has defects affecting methionine synthase directly, whereas the cbIE cell line has defects in the redox proteins. We have also identified a P1173L mutation in the activation domain of methionine synthase in the cbIG cell line WGI505.

Methionine synthase (E.C. 2.1.1.13) is a cobalamin-dependent enzyme that catalyzes two successive transmethylation reactions (1, 2). In one half-reaction, the methyl group from the substrate CH$_3$H$_4$folate$^1$ is transferred to the cob(I)alamin form of the cofactor to generate the intermediate, methylcobalamin (Fig. 1). In the next step, the methyl group is displaced by homocysteine to give methionine and H$_4$folate. Catalysis, however, comes with dangers to the enzyme, since the intermediate, cob(I)alamin, is supernucleophilic and highly reactive and its methylation competes with oxidation. The oxidized enzyme in which the cofactor is in the cob(II)alamin or hydroxycob(III)alamin state is inactive and requires a reductive methylation step for rescue back to the catalytic turnover cycle. Two flavoproteins serve as conduits for the reducing equivalents that are ultimately derived from NADPH in Escherichia coli (3, 4). An analogous system has so far not been described in mammals. Instead, a thiol oxidase activity associated with crude porcine liver methionine synthase and assumed to be an intrinsic property of the enzyme has been postulated to function in reductive activation (5).

In mammals, intracellular cobalamin is processed via a complex multistep pathway. It involves at least three (lysosomal, cytoplasmic, and mitochondrial) and perhaps four (microsomal) cellular compartments and leads to the two cofactor forms, adenosylcobalamin and methylcobalamin, that support enzymatic activity (6, 7). The enzymatic steps involved in this processing pathway remain obscure and have been defined largely by genetic complementation analysis on cell lines from patients with inborn errors of metabolism (8).

Of the various genetically distinct classes of patients with defects in cobalamin metabolism, cbIG and cbIE display isolated functional methionine synthase deficiency (8–10). Both clinically and biochemically, cbIG and cbIE patients have similar phenotypes, displaying megaloblastic anemia, homocystinuria, and hypomethioninemia, which together are consistent with a block in the biosynthetic pathway unique to methylcobalamin formation. In cbIE patients, methionine synthase activity measured in the presence of a high concentration of DTT has been reported to be similar to that in normal fibroblast cell lines (11, 12). In contrast, in cbIG patients, methionine synthase activity has been reported to be either markedly reduced (12, 13) or normal in the presence of high AdoMet concentrations (13). Based on these studies, it has been postulated that cbIG patients may have mutations in methionine synthase (12, 14) or in AdoMet metabolism (13), whereas the cbIE patients have defects in one of the two redox proteins that activates methionine synthase (11). Some of the variability in the reported results could be attributed to the differing degrees of anaerobicity achieved in the in vitro assays in different laboratories. This is a critical issue since the activity of methionine synthase is oxygen-sensitive.

In this study, we report that NADPH can serve as an electron source in the reductive activation of mammalian methionine synthase. Furthermore, fractionation of crude homogenates indicates that at least two redox proteins are required for NADPH-dependent activation of porcine methionine synthase. We have exploited these observations to distinguish between the defective loci in cbIG and cbIE cell lines that exhibit functional methionine synthase deficiency. Our results demonstrate that cbIG and cbIE patients have defects in methionine synthase and in the auxiliary redox protein(s), respectively.

* This work was supported in part by National Institutes of Health Grant DK45776 (to R. B.) and an American Heart Association-Nebraska affiliate predoctoral fellowship (to Z. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CH$_3$H$_4$folate, 5-methyltetrahydrofolate; DTT, dithiothreitol; AdoMet, S-adenosylmethionine; OHChl, hydroxycobalamin; PCR, polymerase chain reaction.
methylene formed/min at 37 °C unless specified otherwise.

**Mutation Detection in cblG WG1505—**This patient has been described previously (13). The mutation and a polymorphism in the methionine synthase gene in this cell line were detected by single strand conformational polymorphism and nucleotide sequence analysis of the cloned cDNA. For the single strand conformational polymorphism experiments, primers P1-S and P2-A, previously used to detect the P1173L mutation in the cblG cell line WG1892, were employed (18). Anomalously migrating bands were excised from the sequencing gel, amplified by PCR, and subjected to direct nucleotide sequencing as described previously (18). The 3′ end of the methionine synthase cDNA from this cell line was amplified by PCR and cloned in the PCR 2.1 TA vector (Invitrogen) as described previously (18). The two alleles were distinguished by MspI digestion (18), and the allele lacking the P1173L mutation was subjected to nucleotide sequence analysis (DNA sequencing facility, University of Nebraska, Lincoln).

**RESULTS**

**NADPH-dependent Activity of Mammalian Methionine Synthase—**Thiols have been implicated as physiological reductants of the mammalian methionine synthase based on the observation that a putative thiol oxidase activity is associated with the crude enzyme (5). We were unable to observe methionine synthase activity when thiols such as glutathione or cysteamine were employed as the sole reductant under aerobic assay conditions (data not shown). In contrast, NADPH supported the酶 activity when thiols such as glutathione or cysteamine were employed as the sole reductant under aerobic assay conditions (data not shown). In contrast, NADPH supported the酶 activity when thiols such as glutathione or cysteamine were employed as the sole reductant under aerobic assay conditions (data not shown). In contrast, NADPH supported the enzyme activity when thiols such as glutathione or cysteamine were employed as the sole reductant under aerobic assay conditions (data not shown). In contrast, NADPH supported the enzyme activity when thiols such as glutathione or cysteamine were employed as the sole reductant under aerobic assay conditions (data not shown). In contrast, NADPH supported the enzyme activity when thiols such as glutathione or cysteamine were employed as the sole reductant under aerobic assay conditions (data not shown).

To determine if the addition of one or more components in the crude homogenate could restore NADPH-dependent activation of pure methionine synthase, DEAE batch chromatography was employed to yield three different fractions of the homogenate. The addition of the 50 and 400 mM potassium Pi fractions was sufficient to sustain NADPH-dependent activity of methionine synthase (Table II). Since reduced pyridine nucleotide oxidases are abundant in liver, the anaerobic assay

**TABLE I**

| Assay Fractions | Methionine formed (µmol) |
|-----------------|--------------------------|
| Homogenate (− NADPH) | 1,443 |
| Homogenate + NADPH | 4,126 |
| Homogenate + NADPH + pure methionine synthase | 8,032 |
| Pure methionine synthase + NADPH | 70 |

a Methionine synthase activity was measured in the anaerobic NADPH assay as described under “Experimental Procedures.”

**TABLE II**

| Eluates | Methionine formed (µmol) |
|---------|--------------------------|
| 50      | 379                      |
| 180     | 312                      |
| 400     | 98                       |
| 50 + 180 + 400 | 1,970                   |
| 50 + 180 | 177                      |
| 180 + 400 | 580                      |
| 50 + 400 | 2,166                    |

2 The specific thiol oxidase activity present in crude porcine liver is inversely related to the specific activity of methionine synthase (Z. Chen and R. Banerjee, unpublished results).
conditions were critical. Under aerobic conditions, NADPH does not support methionine synthase activity even at concentrations as high as 50 mm.

**Activity of Methionine Synthase in cblG and cblE Cell Lines**—Methionine synthase activity was measured under rigorously anaerobic assay conditions using three different sources of reductant (see Table III). A subset of cblG cell lines (WG1670 and WG1671) shows reduced activity under all conditions. A second subset of cblG cell lines (WG1505, WG1308, WG2292) and all the cblE cell lines have methionine synthase activity that is comparable to that of controls when artificial reductants, DTT/OHCbl or titanium citrate, are employed. However, in the presence of NADPH, the activity is approximately 2–30-fold lower in these patient cell lines compared with controls. In the subset of cblG cell lines represented by WG1670 and WG1671, methionine synthase activity was too low to be measured in the NADPH assay, where activity is always lower than in the other two assays.3 The holoenzyme content of methionine synthase ranges from 40 to 100% in these cell lines, and no correlation is observed between the proportion of holoenzyme and the type of defect. This variability in the proportion of holomethionine synthase in cultured cells contrasts with the enzyme being present in a predominantly holoenzyme form in both porcine liver and human plasma (20).

**Complementation of Defect in cblE by cblG**—Methionine synthase activity was measured using the NADPH assay in cblG and cblE cell lines individually and with the combined extracts (see Table IV). One of the cblG cell lines used in this study, WG1655, had very low methionine synthase activity under all conditions, and it was virtually undetectable in the NADPH assay. Mixing cell extracts from two cblE cell lines, WG1575 (9 units/mg) and WG1384 (7 units/mg), did not increase the low specific methionine synthase activity observed with individual cell lines. Similarly, methionine synthase activity was not enhanced by mixing extracts from cblG cell lines WG1655 (1 unit/mg) and WG1505 (3 units/mg). However, when cell extracts from a cblE (WG1575) and a cblG (WG1655) cell line were combined, the methionine synthase specific activity increased to 50 units/mg, which is 5.6-fold above that of the cblE cell line alone.

**Mutation Detection in cblG WG1505**—Single strand conformational polymorphism analysis revealed an anomalous banding pattern (data not shown) previously observed in the same region in the cblG cell line WG1892 (18). Nucleotide sequencing of the excised and PCR-amplified band from WG1505 and comparing the sequence with control cell line samples indicated the presence of a C3804T transition mutation, resulting in a coding sequence change, P1173L. In addition, a likely polymorphism, A3042G, resulting in a coding sequence change, D919G, was detected in the other allele. This polymorphism has been identified previously and was reported to be present in 8 out of 52 control alleles (21). The P1173L mutation is not observed in 160 control cell lines, as described previously (18). It is present in only 1 of the 2 alleles in WG1505, consistent with the polymorphic banding pattern observed by single strand conformational polymorphism in which two of the four bands corresponding to the unaffected allele comigrate with control bands. Restriction digestion of the 359-base pair PCR fragment spanning the C3804T mutation revealed that 2 out of the 5 clones examined contained the mutation, as indicated by the loss of an

3 A third member of this subclass, cblG cell line WG1655, is described in Table IV. The NADPH assay was conducted on this cell line using the maximum possible volume of cell lysate in the assay. Methionine synthase activity was not detected above background levels.

**FIG. 2. Model for interactions between human methionine synthase and its physiological reductive activation system.** The domains involved in catalysis and activation are indicated. Two redox proteins are designated as I and II. Mutations and polymorphisms in cblG cell lines that have so far been described are indicated.

**MspI site (data not shown, but presented previously for WG1892 (18)).**

**DISCUSSION**

Methionine synthase is an oxidation-labile enzyme that can be reductively activated by either DTT/OHCbl or titanium citrate under in vitro assay conditions. In E. coli, two well characterized flavoproteins function to channel reducing equivalents from NADPH to methionine synthase (3, 4). The components of the mammalian physiological activation system are unknown. Our results with highly purified porcine liver methionine synthase indicate that NADPH can serve as a reductant for this enzyme (Table I). Fractionation of the porcine liver homogenate reveals that a minimum of two components are needed to reconstitute the NADPH-dependent activation system (Table II). Thus, the redox activators are separate from methionine synthase, contrary to the suggestion that the activation system may be integral to it (5). These results provide the first evidence that a multicomponent NADPH-dependent system activates mammalian methionine synthase under physiological conditions (Fig. 2).

Homocysteine concentration is increased in 15–40% of patients with coronary, cerebral, or peripheral arterial diseases (22), and it is postulated that elevated homocysteine constitutes an independent risk factor for coronary arterial diseases (23, 24). This has provoked an intense interest in identifying genetic loci and associated polymorphisms/mutations that could either directly or indirectly lead to elevated homocysteine levels. The availability of cell lines with catastrophic mutations affecting methionine synthase activity offers a powerful system that we have exploited to better understand the biochemical events leading to hyperhomocysteinemia. Our results can be interpreted in the framework of the model depicted in Fig. 2. This model predicts that the activity of methionine synthase may be compromised (with attendant hyperhomocysteinemia) by defects in the enzyme itself or in one of the two redox-active proteins. We have therefore employed a trilogy of in vitro assays in which the reductant source is varied in order to construct a biochemical system for distinguishing between impairments that specifically compromise reductive activation versus catalysis.

As is evident in Table III, all the cblE cell lines and a subclass of cblG cell lines (WG1505, WG1308, and WG2292) are indistinguishable from the control cell lines when the artificial reductants, DTT/OHCbl or titanium citrate, are em-
The enzyme activity in these cell lines is higher than previously reported (11, 12, 14) due to the high degree of anaerobiosis achieved in our assays. Under the NADPH-driven assay conditions, marked differences between the patient and control cell lines are observed, ranging from approximately 2-30-fold diminution in activity in the patient group. These results point to the potential diagnostic utility of the NADPH assay for distinguishing cblG and cblE cell lines from controls. The variability of methionine synthase activity in the commonly used DTT/HCBl assay and the similarity of the observed values to those of control cell lines illustrate the difficulty of distinguishing between control and patient cell lines based solely on this assay. The exception to this is the cblG subclass, in which methionine synthase activity is severely reduced under all assay conditions (WG1670 and WG1671). The enzyme activity in these cell lines is >20-fold lower than in the controls. The difference in the holoenzyme content between the two cell lines derived from siblings, WG1670 (44%) and WG1671 (94%), should be viewed with caution since the measured activity is at the lower end of the linear range for this assay.

The biochemical phenotype of cblE and a subset of cblG cell lines that exhibit normal activity with artificial reductants suggests that their impairments specifically affect reductive activation while sparing the catalytic turnover cycle. Under in vitro conditions, this would lead to an accumulation of methionine synthase in an inactive form. However, these assays do not indicate the location of the mutation, i.e., whether it is in methionine synthase or in one of its redox partners. As discussed above, the second subclass of cblG cell lines (represented by WG1670 and WG1671) denoted as “variants” in the literature (16), are noted for their low specific activity under all conditions (Table III). Since cblG cell lines display defects in methionine synthase in the H920D mutation in the B12 binding domain has been reported (12, 14).

This prediction has recently been confirmed by the identification of mutations in the methionine synthase gene in two cblG cell lines (18, 21) after cloning of the human methionine synthase cDNA (21, 25, 26). In cblG WG1892, two mutations (P1173L and ΔI881) in a compound heterozygous state have been found in the peptide domains involved in activation (summarized in Fig. 2). In a second cell line, cblG WG2929, an H920D mutation in the B12 binding domain has been reported (21). In this study, we report the presence of the P1173L mutation in an additional cblG cell line, WG1505. Since the cblG group represents a relatively rare inborn error of metabolism (8, 10), it is surprising that the P1173L mutation has now been found in two patients who are apparently not related.4

The defect in a cblG cell line (79/96) with a biochemical phenotype similar to that of WG1670, WG1671, and WG1655 has also been reported (18). The low specific activity of methionine synthase in cblG 79/96 mirrors very low steady-state levels of methionine synthase mRNA (18). Whereas the precise nature of the defects in these cell lines must await mutation detection analyses, low methionine synthase levels in WG1670 and WG1671 could also explain the low enzyme activity and the virtually undetectable levels of methionine synthase-bound cobalamin in these cell lines as described previously (16). Alternatively, mutations leading to a high $K_m$ for cobalamin could account for the observed phenotype.

Whereas mutation analysis data clearly support methionine synthase as the locus of defects in cblG cell lines, the evidence for cblE cell lines being impaired in the redox proteins has been suggested by biochemical data (11) and genetic complementation studies (14). The somatic cell complementation studies implicate a separate genetic locus. Since the human methionine synthase is a monomeric protein (17), the possibility of intragenic complementation can be ruled out. We have used a biochemical approach complementary to the genetic one to examine if the putative redox protein defect in a cblE cell line can be corrected by provision in trans of normal redox proteins from a cblG cell line that harbors defective methionine synthase. As demonstrated in Table IV, the specific activity of methionine synthase in the cblE cell line WG1575 increases 5.6-fold when measured in the presence of cblG WG1655 in the NADPH-dependent assay. The specific activity of methionine synthase under these conditions (50 units/mg) now falls within the range observed for controls (25–100 units/mg, Table III). In contrast, mixing of two cblG or two cblE cell lines does not enhance the specific activity of methionine synthase. These results demonstrate that the defects in cblE cell lines do not directly involve methionine synthase but rather affect the redox protein(s) that support its activity in vitro (11).

In principle, complementation between two cblE cell lines is possible since a minimum of two redox proteins support the activity of methionine synthase in mammals. We have not examined cblE cell lines other than those reported in Table IV for biochemical complementation (i.e., in the absence of cblG cell lines). However, genetic complementation experiments with cblE cell lines have not indicated intragenic complementation (14). Hence, based on the available biochemical and genetic

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4 Patients WG1505 and WG1892 are both white; the former is from the United Kingdom, the latter from Colorado. Ethnicity data on these patients are not available.
Methionine Synthase Deficiency

TABLE IV
Biochemical complementation between cblG and cblE cell lines

Methionine synthase activity was measured in the anaerobic NADPH assay as described under “Experimental Procedures.” Specific activity is defined as pmol of methionine formed/min/mg of protein at 37 °C.

| Cell line                  | Specific activity |
|----------------------------|-------------------|
| cbl E (WG 1575)            | 9 ± 1             |
| cbl G (WG 1655)            | 1 ± 1             |
| cbl E + cbl E (WG 1575 + WG1384) | 8.2 ± 1.3       |
| cbl G + cbl G (WG 1505 + WG1655) | 3.4 ± 0.3       |
| cbl E + cbl G (WG 1575 + WG1655) | 90 ± 8        |

Acknowledgments—We gratefully acknowledge Helena Leu-Shing and Nora Matiaszuk at McGill University for preparation of the fibroblast cells used in this study.

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evidence, it would appear likely that cblE cell lines have mutations in only one of the redox proteins. We suggest that this may be the proximal reducing partner of methionine synthase rather than the distal enzyme that initially accepts electrons from NADPH. Future characterization of the mammalian redox proteins and mutation detection studies in the respective genes will test the hypothesis that the cblE patients have defects in a single enzyme.

Biochemical complementation between cblE and cblG cell lines can be exploited either independently or in concert with the somatic complementation approach as a useful diagnostic tool to characterize the nature of the defect in a patient with errors in cobalamin metabolism. In addition, this could be an extremely valuable approach for studying other cell types in which methionine synthase defects are suspected. For instance, some tumor cell lines exhibit methionine synthase activity. In the absence of normal redox proteins, the reduction of 5,10-methylenetetrahydrofolate to methylenetetrahydrofolate is impaired. Since reduced methionine synthase activity can result from a cblG- or cblE-like defect, complementation assays with one (cblG) or both cell types would be a useful first step before mutation detection studies are undertaken.

In summary, this study provides the first description of the physiological activation system employed by mammalian methionine synthase. The identities of the individual components await the purification and characterization of these proteins, work that is currently in progress. In addition, we furnish the first unambiguous evidence that cblE cell lines have impairments that are not directly in methionine synthase but in an auxiliary protein that supports methionine synthase activity. This represents an additional locus for genetic defects that can lead to hyperhomocysteinemia.