Purification of Soluble Cytochrome $b_5$ as a Component of the Reductive Activation of Porcine Methionine Synthase*

Zhiqiang Chen and Ruma Banerjee‡

From the Biochemistry Department, University of Nebraska, Lincoln, Nebraska 68588-0664

In mammals, methionine synthase plays a central role in the detoxification of the rogue metabolite homocysteine. It catalyzes a transmethylation reaction in which a methyl group is transferred from methyltetrahydrofolate to homocysteine to generate tetrahydrofolate and methionine. The vitamin B$_5$, cofactor cobalamin plays a direct role in this reaction by alternately accepting and donating the methyl group that is in transit from one substrate (methyltetrahydrofolate) to another (homocysteine). The reactivity of the cofactor intermediate cob(I)alamin renders the enzyme susceptible to oxidative damage. The oxidized enzyme may be returned to the catalytic turnover cycle via a reductive methylation reaction that requires S-adenosylmethionine as a methyl group donor, and a source of electrons. In this study, we have characterized an NADPH-dependent pathway for the reductive activation of porcine methionine synthase. Two proteins are required for the transfer of electrons from NADPH, one of which is microsomal and the other cytoplasmic. The cytoplasmic protein ferredoxin mediates electron transfer to the flavoprotein NADPH-flavodoxin, for electron transfer to methionine synthase. Identification of soluble cytochrome $b_5$, as a member of the reductive activation system for methionine synthase describes a function for this protein in non-erythrocyte cells. In erythrocytes, soluble cytochrome $b_5$ functions in methemoglobin reduction. In addition, it identifies an additional locus in which genetic polymorphisms may play a role in the etiology of hyperhomocysteineemia, which is correlated with cardiovascular diseases.

Homocysteine is a rogue metabolite that is generated by the hydrolysis of $S$-adenosylhomocysteine, the spent form of the ubiquitous methyl group donor $S$-adenosylmethionine. Elevated levels of plasma homocysteine constitute a significant and independent risk factor for cardiovascular diseases (1–7). There are two major metabolic avenues for detoxifying homocysteine in mammalian cells. Transmethylation, catalyzed by methionine synthase or by betaine-homocysteine methyltransferase, salvages homocysteine as methionine, whereas transsulfuration, catalyzed by cystathionine $\beta$-synthase, commits it to degradation (Fig. 1). Of these three enzymes, betaine-homocysteine methyltransferase has a very limited tissue distribution and has been found only in the liver and kidney (8). Mutations in either methionine synthase (9, 10) or cystathionine $\beta$-synthase (11) result in severe hyperhomocystinemia, with attendant early and aggressive occlusive arterial diseases. Noticing the correlation between prominent arterial damage and elevated homocysteine, McCully (12) boldly postulated in 1989 that the vascular alterations could be attributed to high homocysteine concentrations.

Despite the longevity of this hypothesis, little is known about how cells regulate homocysteine levels or about the etiology of homocysteine-dependent vascular changes. Whereas the activity of methionine synthase directly influences intracellular homocysteine concentration, its activity is itself dependent on auxiliary reductases that could influence homocysteine levels indirectly (Fig. 2). This is strongly supported by the existence of the $cblE$ class of patients with an inborn error of cobalamin metabolism resulting in a functional methionine synthase deficiency, albeit the methionine synthase itself is apparently normal (13–15). Biochemical analysis of fibroblast cells from $cblE$ patients indicates that the reductive activation system is compromised. However, the identities of the mammalian proteins that regulate the activity of methionine synthase have not been established.

Methionine synthase (EC 2.1.1.13) is a cobalamin (or vitamin B$_{12}$)-dependent enzyme that catalyzes the transfer of a methyl group from CH$_3$-H$_4$folate to homocysteine (Fig. 2). During this transmethylation reaction, the methyl group is transiently transferred to the cofactor cob(I)alamin, a supernucleophilic and oxidatively labile intermediate, to form methylcobalamin (16, 17). The latter then transfers its methyl group to homocysteine to generate methionine. During catalysis, accidental oxidation of the reactive intermediate, cob(I)alamin, results in leakage of enzyme out of the turnover cycle and its accrual in an inactive cob(II)alamin state. Readmission to the catalytic cycle requires an electron source and $S$-adenosylmethionine as a methyl donor. Under in vitro assay conditions, artificial donors such as titanium citrate or dithiothreitol and hydroxyco-

‡ To whom correspondence should be addressed. Tel.: 402-472-2941; Fax: 402-472-7842; E-mail: rbanerje@unlinfo2.unl.edu.

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† The abbreviations used are: CH$_3$-H$_4$folate, methyltetrahydrofolate; TLCK, N$^2$-p-tosyl-L-lysine chloromethyl ketone; HTP, hydroxyapatite.
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**Component II**

Component has been purified to homogeneity and is soluble cytochrome \( b_5 \), which suggests the identities of the microsomal NADH- and NADPH-dependent oxidases to be cytochrome \( b_5 \) reductase and cytochrome P450 reductase, respectively. Furthermore, purified microsomal cytochrome P450 reductase can reconstitute methionine synthase activity in the presence of soluble cytochrome \( b_5 \). The role of soluble cytochrome \( b_5 \) in the reductive activation of methionine synthase is a novel addition to its so far limited repertoire of known functions. Cytochrome \( b_5 \) represents an additional locus that is a candidate for genetic polymorphisms that may be correlated with mild hyperhomocystinemia.

**EXPERIMENTAL PROCEDURES**

**Materials**

Porcine livers obtained fresh from a slaughterhouse in Crete, Nebraska, were cubed and stored frozen at \(-80 ^\circ C\) until further use. DEAE-cellulose, phenylmethylsulfonyl fluoride, TLCK, dithiothreitol, aprotinin, \( L \)-homocysteine thiolactone, and NADPH were purchased from Sigma. Bio-Gel P-60 gel (medium) was from Bio-Rad. Phenyl-Sepharose (fast flow) was purchased from Amersham Pharmacia Biotech, and HTP was from Calbiochem. \( \text{CH}_3\text{H}_4\text{folate} \) (barium salt; 55 mCi/mmol) was purchased from Amersham Pharmacia Biotech. \( \text{CH}_3\text{H}_4\text{folate} \) (calcium salt) was obtained from Schircks’s Laboratories (Jona, Switzerland).

**Preparation of Microsomes**

Two procedures were employed interchangeably for purifying microsomes. The first protocol (21) represents a slightly larger scale purification in which 300 g of cubed pig liver was placed in 1 liter of 5 mM Tris (pH 7.2) containing 0.25 mM sucrose, 1 mM MgCl\(_2\), 13 mg of trypsin inhibitor, 25 mg of phenylmethylsulfonyl fluoride, 3 mg of TLCK, and 1 ml of aprotinin. The following day, the liver pieces were homogenized in a Waring blender for 3 min at high speed with 1-min intervals to prevent overheating. The homogenate was centrifuged at 12,000 \( \times g \) for 30 min, and the supernatant was filtered through two layers of Miracloth.

**Assay for Component I and II Activities**

Component I and II activities were monitored indirectly by following the activity of methionine synthase. Methionine synthase was purified as described previously (24). The anaerobic NADPH-dependent assay (15, 25) was employed, in which the reductive activation system for methionine synthase was reconstituted by adding fractions to be tested for component II activity to an assay mixture containing microsomes (0.6 mg of total protein) with component I activity and purified methionine synthase. Under these conditions, the activity of methionine synthase is limited by the presence of component II. During the purifi-
fication of component II, typically 200–400 μl aliquots of fractions were added to the assay after being made anaerobic. Similarly, for measuring component I activity, methionine synthase and component II were provided in the assay mixture, and variable amounts of component I were added.

Reconstitution of Methionine Synthase Activity with Purified Cytochrome b₅ and Either Microsomes or Cytochrome P₄₅₀ Reductase

The activity of methionine synthase was reconstituted under in vitro conditions using full-length purified recombinant rat cytochrome P₄₅₀ reductase, which was a generous gift from Thomas Shea and Bettie-Sue Masters (University of Texas Health Science Center, San Antonio, TX). The anaerobic assay mixture contained, in a final volume of 1 ml, 100 mM potassium Pi (pH 7.2), 500 μM homocysteine, 19 μM S-adenosylmethionine, 1 mM NADPH, 250 μM (RS)-5-[14C]CH₃-H₄folate, and the three enzymes methionine synthase, soluble cytochrome b₅ (5.2 μM), and cytochrome P₄₅₀ reductase (0.82 μM) or variable amounts of microsomes as indicated in Table I. The assay was initiated and conducted as described previously (25).

Mass Spectroscopy

Electrospray mass spectrometry was performed on a PLATFORM spectrometer (Micromass) at the Department of Chemistry, University of Nebraska. The protein samples were prepared for mass spectroscopy by exchanging the buffer with water by repeated dilution and concentration of the sample in a Microcon-3 filter (Amicon, Inc.). The protein concentration of the final sample was ~1 mg/ml.

Peptide Sequencing

The protein band was excised from a 15% SDS-polyacrylamide gel and submitted to the University of Nebraska Protein Core Facility for N-terminal and internal peptide sequence analysis.

RESULTS

Purification and Identification of Component II—We have employed a combination of anion-exchange, hydrophobic, and size-exclusion chromatographic steps to purify component II (Table I). Enzyme activity eluted in a broad peak following anion-exchange chromatography on a DEAE-cellulose column (Fig. 3A). Chromatography on two other columns, hydroxyapatite and Bio-Gel P-60 gel filtration, resulted in elution of a single and relatively sharp band containing component II activity (Fig. 3, B and C). Hydrophobic chromatography on a phenyl-Sepharose column resulted in a convenient negative purification since component II bound weakly to the column under the conditions employed. The four-step purification of component II yielded a single protein with a molecular mass of ~14 kDa as estimated on a denaturing SDS-polyacrylamide gel (Fig. 4A). Separation on a calibrated size-exclusion column yielded a slightly larger estimate of 17 kDa for the native protein (data not shown). Hence, component II is a small monomeric protein.

The identity of component II was established unambiguously to be soluble cytochrome b₅ by four independent methods. First, component II comigrated with purified soluble human cytochrome b₅ in 20-fold excess at maximal activation. However, only a low proportion (30%) of the cytochrome b₅ that we isolated had bound heme. We presently do not know whether or not the heme is required for the function of component II; typically observed, indicating that it is a hemeprotein. As shown in Fig. 3 (B and C), the activity of component II coeluted with heme absorption monitored at 413 nm. Purified component II has a typical oxidized cytochrome b₅-like spectrum, with a Soret absorption maximum at 413 nm and a broad absorption band in the 550 nm region (Fig. 6). Reduction with dithionite shifts the Soret band to 423 nm and sharpens the a and b bands at 555 and 525 nm, respectively. The a band is asymmetric with a shoulder at 560 nm (Fig. 6, inset), which is a characteristic of reduced cytochrome b₅ (27). The presence of a b-type heme was also independently confirmed by the pyridine hemochromogen assay (Ref. 28 and data not shown).

Kinetics of Activation—The anaerobic assay employed to monitor component II activity (henceforward referred to as soluble cytochrome b₅) activity during its purification is limited by its concentration. Fig. 7A shows that the activity of methionine synthase was reconstituted with soluble cytochrome b₅ in a saturable manner. A Michaelis-Menten analysis of this data yielded a Kₘ of 0.19 ± 0.05 μM and a Vₘₐₓ of 6.34 ± 0.44 nmol min⁻¹. Based on the concentration of the methionine synthase used in these experiments, we estimate that soluble cytochrome b₅ is in 20-fold excess at maximal activation. However, only a low proportion (~30%) of the cytochrome b₅ that we isolated had bound heme. We presently do not know whether or not the heme is required for the functioning of cytochrome b₅ in this activation system, as discussed below.

Microsomal Localization of Component I—Initial attempts at purifying component I activity from the soluble fraction were unsuccessful. Two observations indicated that component I is membrane-associated. First, component I activity could be precipitated from the liver homogenate at 25% ammonium sulfate saturation, which is typical for many membrane-associated proteins. Second, fractions containing component I activity from pilot chromatography columns that were tested initially were turbid and whitish. The microsomal location of component I was confirmed by the co-purification of glucose-6-phosphatase and component I activities (data not shown).

Reconstitution of Methionine Synthase Activity with Either Microsomes or Purified Cytochrome P₄₅₀ Reductase and Soluble Cytochrome b₅—The activity of methionine synthase in the presence of soluble cytochrome b₅ was dependent on the pres-

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**Table I**

| Procedure       | Volume | Units/ml | Total units | Protein | Total protein | Specific activity<sup>a</sup> | Yield | Purification |
|-----------------|--------|----------|-------------|---------|---------------|-------------------------------|-------|--------------|
| Homogenate      | 2050   | 2655     | 5,442,750   | 27      | 77,080        | 100                           | 99.6  | 9            |
| DEAE-cellulose  | 33     | 15,656   | 516,714     | 24      | 792           | 5.5                           | 652   | 3            |
| HTP             | 60     | 2981     | 178,860     | 3.4     | 204           | 3.3                           | 877   | 12           |
| Phenyl-Sepharose| 6      | 22,440   | 134,640     | 11.8    | 71            | 2.5                           | 1902  | 27           |
| Bio-Gel P-60    | 1.8    | 72,278   | 130,100     | 1.5     | 2.7           | 2.4                           | 48,185| 683          |

<sup>a</sup> Specific activity is expressed as 1 nmol of methionine formed per h/mg of component II.
hydride oxidation to methionine. In utero assay conducted in the presence of dithiothreitol and co-factor NADPH demonstrated that the reductase activity from microsomes could be substituted for the purified enzyme in the standard in vitro assay. The activity of the purified enzyme was measured under the assay conditions described in Table II.

**DISCUSSION**

Mammalian methionine synthase has been the subject of intense interest since it is one of the two cellular enzymes that convert homocysteine to methionine. The reductive activation of methionine synthase has been shown to be sensitive to the presence of dithiothreitol and hydroxycobalamin.

**FIG. 3.** Elution profile of component II activity from three columns used for its purification. A, DEAE-cellulose; B, HTP; C, Bio-Gel P-60 gel filtration. The columns were run as described under "Experimental Procedures." Component II activity was measured as described under "Experimental Procedures." The co-purification of component II activity and heme absorbance on the HTP and Bio-Gel P-60 columns are indicated in B and C, where the open circles represent the absorbance data, and the closed circles represent the activity data.

**FIG. 4.** Purification of component II. A, progressive enrichment of cytochrome b5 during purification of component II activity. Equal amounts of protein (20 mg) were loaded in each lane, separated on a 15\% SDS-polyacrylamide gel, and stained with Coomassie Blue. Lanes 1–5 represent protein obtained from the following steps: homogenate, DEAE-cellulose, HTP, phenyl-Sepharose, Bio-Gel P-60 gel filtration, respectively. Lane 6 has the following molecular mass markers (from top to bottom): phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa; very faint), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

**FIG. 5.** Electrospray mass spectrum of purified component II. 26251

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control homocysteine metabolism in cells. It catalyzes two successive methyl transfer reactions from the substrate CH$_3$-H$_4$folate to the cofactor cob(I)alamin and from methylcobalamin to the second substrate, homocysteine (Fig. 2). The reactivity of the cob(I)alamin intermediate results in its inadvertent escape to an oxidized and inactive enzyme form approximately once every 100–2000 turnovers depending on the assay conditions (20, 29). The oxidized enzyme is rescued to the catalytic cycle in a reductive methylation reaction that employs S-adenosylmethionine and an electron source.

The physiological pathway for electron transfer in bacteria is well studied and involves two flavoproteins, NADPH-dependent flavodoxin reductase and the proximal reducing partner, flavodoxin (19, 20). In mammals, which apparently lack flavodoxin, the nature of the physiological reducing system was unknown. Partially purified porcine methionine synthase was reported to have an associated thiol oxidase activity (30) that was postulated to function in the reductive activation of the mammalian enzyme. However, we found an inverse correlation between specific thiol oxidase and methionine synthase activities during purification of the porcine enzyme, ruling out the presence of an inherent thiol oxidase activity (15).

Early studies on reconstitution of the physiological activation system of porcine methionine synthase in our laboratory revealed that at least two components were required for its activation (15). The first step in the purification of porcine methionine synthase involves anion-exchange batch chromatography and results in the separation of two other fractions that are required to support its activity in the anaerobic NADPH assay.

In this study, we have employed a biochemical reconstitution strategy for elucidating the cellular localization of the two components and have purified one of them to homogeneity. A similar approach has been used previously for identifying activation system components, for instance, for the anaerobic ribonucleotide reductase (31). Chromatography of the crude porcine liver homogenate on DEAE-cellulose yielded two fractions, one of which was turbid and milky in appearance. The particulate nature of this fraction indicated that component I was membrane-associated. The co-purification of component I and glucose 6-phosphatase activities confirmed its microsomal localization.

The second component, cytochrome $b_5$, is soluble and in fact co-purifies with methionine synthase during the first three steps of the published protocol for purifying methionine synthase (24). This accounts for the early observation that a cytochrome $b_5$ with an intense absorption at 413 nm is a “major contaminant of the transmethylase” (32). In the purification protocol reported here that was optimized for soluble cytochrome $b_5$, methionine synthase activity was separated in the second step following chromatography on the HTP column. The isolated protein has been unambiguously identified as soluble cytochrome $b_5$ by several independent methods including precise molecular mass and peptide sequence determinations. Electrospray mass spectrometry revealed a molecular mass of 10,977 Da, which corresponds to a soluble cytochrome $b_5$ extending from residue 2 through 97. It is well known that the

![Fig. 6. UV-visible absorption spectra of oxidized and reduced component II. The oxidized spectrum is of the enzyme as isolated (dashed line). The reduced spectrum was generated by addition of solid sodium dithionite to 0.17 mg/ml protein (solid line). Inset is a blowup of the a-band of reduced cytochrome $b_5$.](image1)

![Fig. 7. Dependence of methionine synthase activity on component II (soluble cytochrome $b_5$) (A) and microsomal concentration (B). The closed circles in A represent the experimental data points, and the solid line represents a fit to the full data set using the Michaelis-Menten equation. The $K_{act}$ for component II obtained from this analysis is 0.19 $\mu$M. MS, methionine synthase.](image2)
The open reading frame in the isolated cDNA is postulated to code for a 24-base pair insert that contains an in-frame stop codon and leads to the translation of a 98-residue-long polypeptide (26). The soluble form of cytochrome P450 reductase has been isolated recently (37). Two functions in methemoglobin reduction in erythrocytes (33). The bacterial flavodoxin and flavodoxin reductases act as an adaptor protein to bring an electron donor anchored in the membrane together with an electron acceptor in the cytoplasmic compartment. Methionine synthase, on the other hand, is a soluble protein in both bacterial and mammalian cells. Hence, nature may have recruited soluble cytochrome b₅ as an adaptor protein to bring an electron donor anchored in the membrane together with an electron acceptor in the cytoplasm (Fig. 9). It is interesting to note that the bacterial flavoproteins flavodoxin and flavodoxin reductases share homologies with the FMN- and FAD/NADPH-binding domains of cytochrome P450 reductase, respectively (38). Thus, during evolution, the two domains may have fused to generate a single protein (39). This was apparently accompanied by the relocation of the flavoprotein from the cytoplasmic to the microsomal compartment. Methionine synthase, on the other hand, is a soluble protein in both bacterial and mammalian cells. Hence, nature may have recruited soluble cytochrome b₅ as an adaptor protein to bring an electron donor anchored in the membrane together with an electron acceptor in the cytoplasm (Fig. 9). It is interesting to note that the bacterial flavoproteins flavodoxin and NADPH-flavodoxin reductase are able to reduce cytochrome P450, the target of cytochrome P450 reductase (38). Cytochrome b₅ was found to stimulate 17α-hydroxyprogrenolone lyase while inhibiting cytochrome P450₁₇α₂-progesterone-17α-hydroxylase activity when the bacterial flavoproteins were employed (40). We have also found that the E. coli flavodoxin and flavodoxin oxidoreductase at high concentrations can activate porcine methionine synthase in an NADPH-de-
pendent reaction. The effect of cytochrome $b_5$ and the characteristics of this heterologous activation system will be further examined in the future.

Severe thermodynamic constraints limit the direct involvement of cytochrome $b_5$ in electron transfer in a pathway leading from NADPH to methionine synthase. The redox potential of cytochrome $b_5$ is positive, in the range of $-20$ mV to $+20$ mV (41–43). The redox potential for the cob(II)alamin/cob(I)alamin couple has been estimated to be $-490$ mV (44) and $-520$ mV (45). Activation of oxidized methionine synthase is postulated to occur via a thermodynamically uphill reduction of cob(I)alamin to cob(II)alamin rendered favorable by the irreversible trapping of cob(I)alamin in an exergonic methylation reaction (Equation 1),

$$\text{Cob(II)alamin} + e^- \rightarrow \text{Cob(I)alamin}$$

where AdoMet is $S$-adenosylmethionine and MeCbl is methylcobalamin. Coupling of reduction to a highly favorable alkylation reaction explains why reductive methylation of methionine synthase is observed even at an ambient potential as high as $-82$ mV (45). Thus, at least in principle, cytochrome $b_5$ could serve as a proximal electron donor to the porcine methionine synthase. This would be facilitated if the redox potentials of either the heme or cobalamin (or both) are significantly perturbed in a methionine synthase-cytochrome $b_5$ complex. However, it is unlikely that the magnitude of the shift, if any, would span the almost $0.5$ V difference between the two redox couples.

Instead, soluble cytochrome $b_5$ may function to facilitate complex formation between the microsomal redox donor and its soluble redox acceptor, methionine synthase. A similar role for the membrane-associated cytochrome $b_5$ has recently been revealed in two related systems. Cytochrome $b_5$ was postulated to donate the second electron to cytochrome P450 during the catalytic cycle (46). A similar discrepancy between the redox potential of the putative donor (cytochrome $b_5$) and acceptor (e.g. cytochrome P4503A4, approximately $-310$ mV) pertains to this system (47). It has been demonstrated recently that apocytochrome $b_5$ can replace holocytochrome $b_5$ in augmenting the oxidation activity of cytochrome P4503A4 and the 17,20-lyase activity of cytochrome P450c17 (47, 48). These studies demonstrate unambiguously that the role of cytochrome $b_5$ in these systems does not depend on its redox activity.

In summary, we have purified soluble cytochrome $b_5$, which is one of two proteins in the two-component system for activation of porcine methionine synthase. The NADPH oxidoreductase in this redox pathway is associated with the microsome and could be the well studied cytochrome P450 reductase whose three-dimensional structure has recently been determined (49). This hypothesis is supported by the reconstitution of methionine synthase activity by purified cytochrome P450 reductase and soluble cytochrome $b_5$. A number of catastrophic mutations in enzymes that either directly (viz. cystathionine $\beta$-synthase and methionine synthase) or indirectly (methyltetrahydrofolate reductase) influence homocysteine homeostasis have been described. These are rare, however, and are inherited as autosomal recessive inborn errors of metabolism. Over the past few years, there has been an intense interest in identifying polymorphisms in genes that influence homocysteine levels and that either alone or in concert with the environment, viz. nutritional status, may constitute a risk for hyperhomocysteinemia. A fairly prevalent polymorphism in methyltetrahydrofolate reductase has been identified that may be correlated with mild hyperhomocysteinemia (50). Soluble cytochrome $b_5$ represents an additional and newly identified target in the homocysteine metabolic pathway for assessing correlations between potential genetic polymorphisms and propensity for cardiovascular diseases.

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