Redundancy in the Pathway for Redox Regulation of Mammalian Methionine Synthase

REDUCTIVE ACTIVATION BY THE DUAL FLAVOPROTEIN, NOVEL REDUCTASE 1*

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Methionine synthase is an essential cobalamin-dependent enzyme in mammals that catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine to give tetrahydrofolate and methionine. It is oxidatively labile and requires for its sustained activity an auxiliary repair system that catalyzes a reductive methylation reaction. Genetic and biochemical studies have demonstrated that the soluble dual flavoprotein oxidoreductase, methionine synthase reductase, serves as a redox partner for methionine synthase in an NADPH-dependent reaction. However, three reports suggest the possibility of redundancy in this redox pathway. First, a hyperhomocysteinemic patient has been reported who has an isolated functional deficiency of methionine synthase but appears to be distinct from the cbfE and cbfG classes of patients with defects in methionine synthase reductase and methionine synthase respectively. Second, another dual flavoprotein oxidoreductase with significant homology to methionine synthase reductase, NR1, has been described recently, but its function is unknown. Third, methionine synthase can be activated in vitro by a two-component redox system comprised of soluble cytochrome b5 and P450 reductase. In this study, we demonstrate a function for human NR1 in vitro. It is able to full activate methionine synthase in the presence of soluble cytochrome b5 with a Vmax of 2.8 ± 0.1 μmol min⁻¹ mg⁻¹ protein, which is comparable with that seen with methionine synthase reductase. The KactNR1 is 1.27 ± 0.16 μM, and a 20-fold higher stoichiometry of reductase to methionine synthase is required for NR1 versus methionine synthase reductase, suggesting that it may represent a minor pathway in the cell, assuming that the two proteins are present at similar levels.

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The abbreviations used are: CH3-H4folate, 5-methyl tetrahydrofolate; H4folate, tetrahydrofolate; NR1, novel reductase 1; AdoMet, S-adenosylmethionine; GST, glutathione S-transferase; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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of soluble cytochrome b₅-dependent reductive activation system that had been described previously using a functional reconstitution approach in our laboratory (9).

EXPERIMENTAL PROCEDURES

Materials—Glutathione-Sepharose 4B, Sephacryl S200, and (6-R, S)-5-\textsuperscript{14}CH₃H₄folate (barium salt, 55 mCi/mmol) were purchased from Amersham Biosciences. All other chemicals were from Sigma, and restriction enzymes were from Invitrogen unless stated otherwise. (6-R, S)-CH₃H₄folate (calcium salt) was obtained from Schircks’s Laboratoires (Jona, Switzerland). Porcine livers were obtained fresh from a slaughterhouse in Crete, NE, cubed, and stored frozen at −80 °C until further use.

Cloning the cDNA Encoding Human NR1—The cDNA encoding NR1 was cloned by PCR, using the following nondegenerate oligonucleotides based on the published sequence (10): forward, 5'-GCCGAAATCTAGC-CCAGGCCGGCAGCTTGTG-3'; and reverse, (5'-CCCTCGGACTCGGC-CCAGCTTCTGTCGTCG-3'). The sequences in bold letters correspond to EcoRI and XhoI restriction sites that were designed into the primer sequences. The gene was generated using the I.M.A.G.E. consortium clone 4869561 obtained from ATCC (Manassas, VA) as a template. Following amplification of this cDNA using \textit{Pfu} polymerase (Stratagene), the gene was ligated into the TOPO-TA vector (Invitrogen) and sequenced to confirm that no PCR-induced errors had been introduced. The conditions for PCR amplification were as follows: denaturation at 98 °C for 1 min followed by 25 cycles of the sequence 98 °C, 1 min, 65 °C, 1 min, 72 °C, 4 min, followed by extension at 72 °C for 10 min. Subsequently, the 1.8-kb insert containing the open reading frame for NR1 was excised using the EcoRI and XhoI restriction sites and subcloned in-frame with the GST-coding sequence in the pGEX-4T1 vector (Amersham Biosciences). The resulting plasmid, pHONR1, was transformed into the \textit{E. coli} strain BL21(DE3) for expression studies.

Expression of Recombinant NR1—An overnight culture of \textit{E. coli} containing the expression construct was grown at 37 °C in Luria broth medium containing ampicillin (100 μg/ml). One liter of modified Terrific broth (20 g of yeast extract, 10 g of bactotryptone, 4 ml of glycerol, 4.33 g of Na₂HPO₄, and 2.65 g of KH₂PO₄), with 100 μg/ml ampicillin was inoculated with 10 ml of overnight culture and grown at 37 °C. When the culture reached an A₆₀₀ nm of ~1.0, IPTG was added to a final concentration of 0.1 mM and the temperature was switched to 25 °C. The cells were grown further overnight, pelleted by centrifugation at 5,000 × g for 20 min and stored at −80 °C until further use.

The frozen cells were thawed and resuspended in GST wash/bind buffer (4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol) containing Complete\textsuperscript{TM} protease inhibitor tablets. Cell lysis was achieved by lysozyme addition (200 μg/ml) and sonication (7 × 20-s bursts with 5-min pause intervals at a power setting of 7), using a Misonix Sonicator XL2020 (Misonix Inc.). The sonicate was centrifuged at 15,000 × g for 60 min to pellet cell debris and insoluble matter. The resulting supernatant was loaded on a glutathione-Sepharose 4B column, previously equilibrated with GST wash/bind buffer. Nonspecifically bound proteins were washed from the column with 1 liter of GST wash/bind buffer, and the GST-fused NR1 protein was eluted with a 10-mM glutathione solution in 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol. The GST tag was removed by limited proteolysis with thrombin. The buffer was exchanged to 50 mM potassium phosphate buffer, pH 7.2, by overnight dialysis of NR1 at 4 °C. The enzyme was further purified by anion exchange chromatography on a 150 × 25 mm POROS Hq-10 column (PerSeptive Biosystems) using a linear gradient ranging from 50 to 500 mM NaCl in 50 mM potassium phosphate buffer, pH 7.2, and a flow rate of 10 ml/min. NR1-containing fractions were pooled, concentrated, and further purified by affinity chromatography on a 2.5'ADP-agarose column as described (10). The concentration of purified recombinant NR1 was determined spectrophotometrically, using a molar extinction coefficient of 21,800 at 379 nm. 480 nm. ~0.8 mg of NR1 was obtained per liter of culture.

Peptide Sequencing—The NR1 protein band was excised from a 10% SDS-polyacrylamide gel and submitted to the Protein Core Facility (University of Nebraska, Lincoln) for N-terminal sequencing.

Flavin Determination and Spectral Analysis—The flavin cofactor content was determined as previously described (11).

Methionine Synthase Activation Assay—The NADPH-dependent methionine synthase assay was adapted from a published method (16) and monitors the transfer of the \[\text{[14C]}\text{methyl group from CH}_3\text{-H}_4\text{folate to the product, methionine. The assay mixture contained} \]
phosphate buffer, pH 7.2, 100 mM potassium chloride, 500 µM homocysteine, 19 µM AdoMet, 250 µM (6R, S)-5-[3H]CH$_3$H$_2$folate (~2000 dpm/nmol), 5 µM soluble cytochrome b$_5$, 1 mM NADPH, and the indicated amounts of methionine synthase and NR1 in a total volume of 1 ml. Reagents were made anaerobic by purging with nitrogen and added to stoppered assay vials with gas-tight syringes. The mixture lacking CH$_3$H$_2$folate was preincubated at 37 °C for 5 min. The reaction was initiated with CH$_3$H$_2$folate, incubated for 10 min at 37 °C, and terminated by heating at 98 °C for 2 min. The assay mixture was immediately placed on ice for 2 min and then passed through a small (0.5 × 6 cm) column of Dowex 1X8–200 (chloride form). The column was washed with 2 ml of water, and the eluate was collected in a scintillation vial.

Scintillation fluid (10 ml) was added to the aqueous sample, and the eluate was counted in a Beckman LS-9500 liquid scintillation counter. All reported values are corrected for the counts observed in control assays run in parallel from which NR1 was omitted.

Ionic strengths were calculated as described by Perrin and Dempsey (17) to determine the concentration of KCl necessary to achieve the desired ionic strength in the chosen buffer. The ionic strength, I, of a solution is given by Equation 1, where $c_i$ is the concentration of each type of ion (in mols/liter) and $z$ is its charge.

$$I = \frac{1}{2} \sum (c_i z^2)$$

The ionic strength dependence of the methionine synthase activation assay was determined in 50 mM potassium phosphate buffer, pH 7.2, to which KCl was added to give ionic strengths ranging from 120 to 820 mM.

**Cytochrome c Reduction Assay**—Reduction of cytochrome c was monitored by measuring the absorbance change at 550 nm as described in Ref. 10.

**Purification of Methionine Synthase and Soluble Cytochrome b$_5$**—Porcine methionine synthase and soluble cytochrome b$_5$ were purified from pig liver as described previously (9, 18). The specific activity of the porcine methionine synthase used in these studies was 1.5 µmol min$^{-1}$ mg$^{-1}$ in the standard in vitro assay using the artificial reductants, diithiothreitol and B$_{12}$.

**RESULTS**

**Purification of Recombinant Human NR1**—Previous studies have reported the use of a baculovirus system for expression of full-length human NR1 (10) and an E. coli system for expression of its component FAD/NADPH- and FMN-binding domains (19). To facilitate studies with the full-length NR1 protein, we expressed NR1 heterologously in E. coli as a fusion protein with GST. A three-step purification involving two affinity columns, GST-Sepharose and 2′5′ ADP-agarose, and a limited proteolysis step to remove the GST tag were employed (Fig. 3). Recombinant human NR1 was obtained in >95% purity and migrated with a molecular mass of 68 kDa, consistent with the predicted molecular mass of 67 kDa (10). The N-terminal peptide sequence of the purified protein (GSTSNGRQCAEFMFSPLLV, where the underlined residues are of NR1 and the preceding amino acids are in the linker following the thrombin cleavage site) confirmed the identity of recombinant NR1.

**Properties of Recombinant NR1**—The UV-visible absorption spectrum of NR1 displayed features that are typical of oxidized flavoproteins with absorbance maxima at 380, 450, and a shoulder at 480 nm (Fig. 4). The absorbance ratio of the 270 nm (protein) to 450 nm (flavin) peaks was 5.06, consistent with the presence of highly pure protein. Addition of NADPH under aerobic conditions resulted in a decrease in the absorption of the oxidized flavin peaks at 380 and 460 nm and the appearance of long wavelength absorption with a peak at 585 nm and shoulder at 626 nm, consistent with the formation of an air-stable blue semiquinone (Fig. 4, inset). HPLC analysis of the flavins bound to NR1 revealed the presence of stoichiometric FMN and FAD in a 1:1 molar ratio. In these respects, the properties of the human NR1 expressed in E. coli are indistinguishable from those reported previously for the protein isolated from an insect cell culture (10).

NR1, like cytochrome P450 reductase and methionine synthase reductase, is able to reduce cytochrome c, providing a convenient measure of the activity of the protein. Recombinant human NR1 purified from E. coli displayed a cytochrome c reductase activity of 1.98 µmol min$^{-1}$ mg$^{-1}$, which is slightly higher than the value of 1.2 µmol min$^{-1}$ mg$^{-1}$ reported previously for protein isolated from insect cells (10). Thus, by all these measures, human NR1 purified from E. coli and insect cells are indistinguishable.

**Activation of Mammalian Methionine Synthase by NR1**—Recombinant human NR1, unlike methionine synthase reductase, was unable to reactivate porcine methionine synthase in the presence of NADPH and AdoMet (Table I). Surprisingly, addition of soluble cytochrome b$_5$ led to full methionine synthase activity at levels supported by methionine synthase reductase alone and greater than the in vitro activation system, which is seminanaerobic.

During the reactivation reaction, protein-protein interactions between methionine synthase, NR1, and soluble cytochrome b$_5$ are expected to occur and are likely to be sensitive to the ionic strength of the assay mixture. Reactivation of methionine synthase by NR1-soluble cytochrome b$_5$ is maximal between 120–220 mM and declines steeply at higher ionic strengths (Fig. 5). In contrast, reactivation by methionine synthase reductase shows more of a bell-shaped dependence and is maximal at an ionic strength of 220 mM.

**Fig. 3.** Purification of recombinant NR1. Comparative SDS-PAGE of protein samples obtained from sequential purification steps: eluate from glutathione-Sepharose column (lane 2); after limited proteolysis with thrombin (lane 3); after POROS HQ10 anion exchange chromatography and 2′5′-ADP agarose affinity chromatography (lane 4). An equal amount of protein (10 µg) was loaded in each lane. Lanes 1 and 5 contain high molecular mass, and their sizes in kDa are indicated on the right.

**Fig. 4.** UV-visible absorption spectrum of NR1. The spectrum of purified recombinant NR1 (8.0 µM) in 50 mM potassium phosphate buffer, pH 7.2, is shown. The ratio of $A_{270}/A_{450} = 5.06$. The inset presents the spectrum of an aerobic solution of NR1 (6.5 µM in 50 mM potassium phosphate buffer, pH 7.2, solid line) to which 65 µM NADPH (final concentration) was added (dashed line).
isolated functional deficiency in methionine synthase, consistent with the essential role played by this housekeeping synthase in transgenic mice results in embryonic lethality (5), and developmental delay (4). Genetic disruption of methionine synthase has pleiotropic clinical consequences, including hyperhomocysteinemia with attendant severe cardiovascular problems, hypomethioninemia, megaloblastic anemia, and developmental delay (4).

Simultaneously, it plays a key role in critical biochemical transformations, including steps involved in purine biosynthesis. S-adenosylhomocysteine and one-carbon donors serve as a gateway enzyme that makes the circulating form of folic acid, CH$_3$-H$_4$folate, available intracellularly to support critical biochemical transformations, including steps involved in purine biosynthesis. Simultaneously, it plays a key role in
dysfunction of methionine synthase, which may be physiologically relevant.

**TABLE I**

| Assay conditions* | Methionine synthase activity $\mu$mol min$^{-1}$ mg$^{-1}$ |
|-------------------|----------------------------------------------------------|
| -NR1              | 0                                                        |
| -NADPH            | 0                                                        |
| -soluble cytochrome $b_5$ | 0                                                   |
| NR1 + NADPH + soluble cytochrome $b_5$ | 2.8 ± 0.1 |
| Standard assay (dithiothreitol + B$_{12}$)$^b$ | 1.5 ± 0.2 |

*The anaerobic NADPH assay described under “Experimental Procedures” was employed for the first four entries, and the assay mixtures contained the same amount of porcine methionine synthase. In the standard assay, NADPH was replaced with dithiothreitol and B$_{12}$ as described under “Experimental Procedures.”

$^b$The activity in the standard assay is lower because it is performed under semianaerobic conditions.

**DISCUSSION**

Methionine synthase is an essential gene in mammals and serves as a gateway enzyme that makes the circulating form of folic acid, CH$_3$-H$_4$folate, available intracellularly to support critical biochemical transformations, including steps involved in purine biosynthesis. Simultaneously, it plays a key role in removal of homocysteine, a sulfur-containing amino acid that is generated by hydrolysis of S-adenosylhomocysteine and one that is toxic when present at elevated levels (3). Dysfunction of methionine synthase has pleiotropic clinical consequences, including hyperhomocysteinemia with attendant severe cardiovascular problems, hypomethioninemia, megaloblastic anemia, and developmental delay (4). Genetic disruption of methionine synthase in transgenic mice results in embryonic lethality (5), consistent with the essential role played by this housekeeping enzyme in folate and sulfur metabolism.

The issue of whether or not Nature has devised redundancy in the reductive activation pathway necessary to sustain the activity of this essential enzyme is an interesting and clinically important one. The genetic evidence until recently has pointed strongly to the existence of only two loci, $cb$ and $cb$E, that are correlated with isolated functional deficiency in methionine synthase. There is unequivocal evidence that these represent methionine synthase and methionine synthase reductase, respectively, because mutations in these genes have been found in patient cell lines (8, 12, 20–22). However, a patient with an isolated functional methionine synthase deficiency has been reported (13) whose defect is not corrected by the addition of folic acid, $M$ and a $K$$_{act}$ value for NR1 of 1.27 ± 0.16 $\mu$M and a $V$$_{max}$ of 2.83 $\mu$mol min$^{-1}$ mg$^{-1}$ are obtained from the kinetic analysis (Table I). Maximal activity of methionine synthase by NR1-soluble cytochrome $b_5$ ($\rightarrow$) and by methionine synthase reductase (–). Data for methionine synthase reductase is from Ref. 11.

Reductive activation of methionine synthase by NR1 displays saturation kinetics (Fig. 6), and a $K$$_{act}$ value for NR1 of 1.27 ± 0.16 $\mu$M and a $V$$_{max}$ of 2.83 $\mu$mol min$^{-1}$ mg$^{-1}$ are obtained from the kinetic analysis (Table I). Maximal activity of methionine synthase was observed at a stoichiometry of 67 moles NR1/mole methionine synthase (Table II).

**FIG. 6.** Dependence of methionine synthase activity on the concentration of NR1. Inset, the experimental data were fitted to the Michaelis-Menten equation and yielded a $K$$_{act}$ value for NR1 of 1.27 ± 0.16 $\mu$M and a $V$$_{max}$ of 2.8 ± 0.1 $\mu$mol min$^{-1}$ mg$^{-1}$.

We have addressed these questions in the present study and demonstrate that NR1 is able to fully activate porcine methionine synthase in an NADPH-dependent reaction. However, unlike methionine synthase reductase, its ability to reactivate methionine synthase is completely dependent on the presence of soluble cytochrome $b_5$ (Table I). This observation suggests that the two-component reductive activation system that we had initially described based on a biochemical fractionation-reconstitution approach (9) could have been soluble cytochrome $b_5$ and NR1. It is, of course, puzzling that this approach did not yield methionine synthase reductase, but this is most likely because the FMN is loosely bound and tends to be lost during purification leading to inactive protein (11). Because reduced FMN is efficacious in replacing dithiothreitol and B$_{12}$ in the standard in vitro assay (24), this cofactor could not be added during the initial stages of purification, likely precluding detection of methionine synthase reductase-dependent reactivation of methionine synthase.
Although the physiological relevance of methionine synthase reductase as a redox partner for methionine synthase is unquestionable, the relevance of the NR1-soluble cytochrome b₅ pathway is an open one. Defects in the reactivation pathway have been reported in only a very few patients, and the majority have been demonstrated to harbor mutations in methionine synthase reductase (12). Only one thus far, albeit poorly characterized, appears to be different (13). Biochemically, there is a significant difference in the efficacy of the two systems as revealed by the large differences in the stoichiometries required for full activation of methionine synthase, i.e. 3:1 for methionine synthase reductase and 67:1 for NR1 (Table II). In the absence of information on the relative intracellular concentrations of methionine synthase versus NR1, it is difficult to ascertain whether the NR1 pathway could represent a functionally significant one for methionine synthase.

The pathway for electron transfer in both methionine synthase reductase and NR1 is expected to proceed from NADPH through FAD to FMN to the redox partner, methionine synthase. The potential of the cob(II)alamin/cob(I)alamin redox couple is very low in solution (25) and in bacterial methionine synthase (26). Assuming that the cobalamin in the human enzyme, which is 55% identical to the E. coli enzyme (21, 15), has a similarly low potential, reactivation of methionine synthase by either dual flavoprotein poses a thermodynamic challenge. The potentials of the four one-electron redox couples of the FMN and FAD cofactors in methionine synthase reductase (14) and NR1 (19) have been reported recently. Interestingly, the redox potential of the FMN semiquinone/hydroquinone couple in NR1 is more negative (305 mV) than the corresponding couple in methionine synthase reductase (317 mV), which would make it a better reductant for cob(II)alamin. In either case, the unfavorable reduction of methionine synthase would be coupled to a highly exergonic methylation by AdoMet, which drives the reductive methylation reaction in the forward direction (26).

In summary, we have demonstrated that NR1, a novel soluble dual flavoprotein oxidoreductase of previously unknown function is able to reactivate mammalian methionine synthase in vitro in the presence of soluble cytochrome b₅. However, given the large excess of NR1 to methionine synthase that is required for detectable activation, it is most probable that under physiological conditions this pathway plays a minor role, if any, in reductive activation of methionine synthase. It would be of interest, however, to conduct mutational analysis of the NR1 gene in the patient with a functional deficiency of methionine synthase, where the affected locus has been identified to be distinct from the cblG and cblE classes.

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