Heterologous High Level Expression, Purification, and Enzymological Properties of Recombinant Rat Cobalamin-dependent Methionine Synthase*

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Rat methionine synthase was expressed chiefly as apoenzyme in recombinant baculovirus-infected insect cells (Yamada, K., Tobimatsu, T., and Toraya, T. (1998) Biosci. Biotech. Biochem. 62, 2155–2160). The apoenzyme produced was very unstable, and therefore, after complexation with methylcobalamin, the functional holoenzyme was purified to homogeneity. The specific activity and apparent $K_m$ values for substrates were in good agreement with those obtained with purified rat liver enzyme. The electronic spectrum of the purified recombinant enzyme resembled that of cob(II)alamin and changed to a methylcobalamin-like one upon incubation of the enzyme with titanium(III) and S-adenosylmethionine. The rate of oxidative inactivation of the enzyme in the absence of S-adenosylmethionine was slower with a stronger reducing agent like titanium(III). The nucleotide moiety, especially the phosphodiester group, was shown to play an important role in the binding of the coenzyme to apoprotein and thus for catalysis. Upon incubation with the apoenzyme in the absence of a reducing agent, cyano- and aquacobalamin were not effective or were effective only slightly in reconstituting holoenzyme. Ethyl- and propylcobalamin formed inactive complexes with apoenzyme, which were converted to holoenzyme by photolytic activation. Adenosylcobalamin was not able to form a complex with apoenzyme, which was convertible to holoenzyme by photoirradiation.

Methionine synthase (5-methyltetrahydrofolate: homocysteine methyltransferase) is a Cbl$^1$ (B$_2$)$_2$-dependent enzyme that catalyzes the methyl transfer reaction from MeH$_4$F to homocysteine forming H$_4$F and methionine. MeCbl is involved as an intermediate in the two-step methyl transfer reactions, that is, cob(II)alamin is first methylated by MeH$_4$F forming MeCbl and H$_4$F, and MeCbl is successively demethylated by homocysteine forming methionine and regenerating cob(II)alamin. Because intermediate cob(II)alamin is a supernucleophile and easily oxidizable, the enzyme undergoes oxidative inactivation under aerobic conditions (1). For reactivation of the inactivated enzyme, AdoMet and a one-electron reducing system are required. The physiological partners transferring a reducing equivalent from NADPH to the enzyme are two flavoproteins in Escherichia coli (2). Recently, it has been reported that at least two auxiliary redox proteins are needed for physiological reactivation of mammalian enzyme with NADPH (3). Human cDNA for methionine synthase reductase has been cloned (4). Three-dimensional structures of Cbl- and AdoMet-binding domains of the E. coli methionine synthase were recently analyzed using respective fragments obtained by partial digestion with trypsin (5, 6). It was revealed that Cbl undergoes a marked conformational change upon binding to the enzyme (5). That is, upon binding to apoenzyme, the 5,6-dimethylbenzimidazole ligand coordinating to the cobalt atom in the free cofactor is displaced from the cobalt atom, and instead, the imidazole group of a certain histidine residue of the protein is ligated to it. Unique motifs for cobalamin binding in the so-called “base-off” mode as well as that for AdoMet binding were also reported (5, 6). cDNA cloning of the human (7–9) and rat (10) methionine synthases revealed that both enzymes are very similar to the E. coli (11) enzyme in their primary structures. All the motifs for the Cbl and AdoMet binding are completely conserved in the rat enzyme, and amino acid sequences around these motifs are also highly conserved. These suggest that mammalian methionine synthases also bind Cbl in the base-off mode with histidine ligation and catalyze the reaction by essentially the same mechanism as that for the E. coli enzyme, although slight differences between the E. coli (12) and mammalian (13) methionine synthases in cofactor specificities were reported previously.

To elucidate the mechanism for mammalian methionine synthase, a large quantity of enzyme is necessary. Because the mammalian tissues contain the enzyme only in an extremely low level, we attempted to establish a method for high level expression of recombinant rat methionine synthase in insect cells using a baculovirus expression system (10). In this paper, we report heterologous expression, purification, and enzymological properties of recombinant rat methionine synthase.

EXPERIMENTAL PROCEDURES

Materials

Crystalline McCbl and AdoCbl were a gift from Eisai Co., Ltd. (Tokyo, Japan). aqCbl was prepared by photolysis of AdoCbl. EtCbl and PrCbl were prepared by reactions of cob(II)alamin with ethyl bromide and n-propyl iodide, respectively, and MeCbl was prepared by reaction of cob(II)alamin with methyl iodide (14). Cyanocobalamin and methylphosphate synthesized as reported previously (15) was converted to MeCblP-Me by the same method. Titanium(III) citrate was prepared

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1 The abbreviations used are: Cbl, cobalamin; AdoMet, S-adenosyl-L-methionine; McCbl, methylcobalamin; AdoCbl, adenosylcobalamin; aqCbl, aquacobalamin; CN-Cbl, cyanocobalamin; EtCbl, ethylcobalamin; PrCbl, propylcobalamin; MeCbl, methylcobalamin; MeCblP-Me, methylcobalamin methyl phosphate; MeH$_4$F, 5-methyltetrahydrofolate; H$_4$F, tetrahydrofolate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
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from TiCl$_4$ (Nacalai tesque) according to the method of Zehnder and Wuhrmann (16). [$methyl^{-14}$C]Me$_2$F$_2$ was purchased from Amersham Pharmacia Biotech. Me$_3$F$_2$ barium salt) and AdoMet (chloride salt) were obtained from Sigma. Homocysteine was purchased from Aldrich.

Expression of Rat Methionine Synthase cDNA in E. coli Cells Using a T7 Expression System

The cDNA containing the coding region of rat methionine synthase (10) was inserted into plasmid vector pRK172 (17) downstream of T7 promoter. The resulting plasmid was transformed to E. coli BL21 (DE3) and expression of the methionine synthase cDNA was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside to the growing culture. The Sf cells harvested were used for expression of recombinant rat methionine synthase. Sf21 (Spodoptera frugiperda) insect cells were maintained at 27.5 °C without supplementation of CO$_2$. The recombinant baculovirus containing the coding region of rat methionine synthase cDNA was used as reported previously (10). The media used for insect cell growth were SF-900 II SFM (Life Technologies, Inc.) and EXCELL 400 (JRH Bioscience). Antibiotics, such as gentamicin sulfate and amphotericin B, were added to the medium. Both monolayer culture and spinner culture were used for expression of recombinant rat methionine synthase. Sf21 cells infected with the recombinant baculovirus were cultured with or without added CN-Cbl. The maximal production of the recombinant protein was usually obtained in 3 days post-infection for monolayer culture and in 3–4 days for spinner culture. The Sf cells were harvested with phosphate-buffered saline and stored at ~80 °C until use.

Enzyme and Protein Assays

Methionine synthase activity was determined by two methods: a standard assay and an anaerobic assay. The standard assay according to the method of Frasca et al. (18) was performed as follows. The mixture contained 0.56 mM homocysteine, 40 μM AdoMet, 25 mM DTT, 50 μM aqCbl, an appropriate amount of enzyme, and 0.2 M potassium phosphate buffer (pH 7.2). After the mixture was preincubated at 37 °C for 5 min, reaction was started by adding 0.16 mM [14C]MeH$_4$F (specific activity, 2,000 dpm/nmol) to a final volume of 250 μl. After 10 min at 37 °C, the reaction was terminated by quick chilling and dilution with 2 volumes of ice-cold water. The amount of methionine produced was determined by passing through a Dowex 1(Cl-) column and counting $^{14}$C radioactivity. The anaerobic assay was performed as described by Chen et al. (19) using Ti(III) citrate as a reducing agent. The conditions were the same as the standard assay, except that Ti(III) citrate was added to a final concentration of 2 mM instead of aqCbl/DTT, and that the reaction was carried out under a H$_2$ gas atmosphere. One unit of methionine synthase was defined as the amount of enzyme activity that catalyzes the formation of 1 pmol of methionine/min at 37 °C. The total activity and holoenzyme activity were determined with and without added MeCbl. This solution was used as the crude extract.

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Expression of Recombinant Rat Methionine Synthase in E. coli Cells and Preparation of Antiserum against the Rat Enzyme—We first attempted to obtain functional recombinant enzyme using procaryotic expression systems. When the rat methionine synthase cDNA was placed under control of either the tac promoter or T7 promoter, the product of the cDNA was expressed as a catalytically inactive polypeptide forming inclusion bodies. Therefore, the expression of functional rat methionine synthase in E. coli cells was not successful. To use this inactive polypeptide as an antigen for preparation of rabbit anti-rat methionine synthase antibody, the polypeptide overexpressed in an insoluble fraction was extracted from cell lysate with 0.15 M Tris-HCl buffer (pH 7.5) containing 8 M urea. The extract was subjected to SDS-PAGE, and the band corresponding to methionine synthase polypeptide was cut out to recover the polypeptide by electro-elution. A mixture of this purified polypeptide and a Freund's complete adjuvant was injected into the back of the rabbit for immunization. Seven days after the second injection, the rabbit was killed to collect antiserum against rat methionine synthase. Western blot analysis of rat liver cytosolic fraction with this antiserum gave only a single band with a molecular weight of 140,000 which corresponded to methionine synthase (data not shown).

Expression of Recombinant Rat Methionine Synthase in Insect Cells—The functional expression of rat methionine synthase was achieved in insect cells using a baculovirus expression system. In monolayer culture, the maximum level of expression of the recombinant enzyme was obtained in 3 days postinfection (10). Addition of CN-Cbl to the medium at a concentration up to 25 μM did not increase the amount of enzyme produced (data not shown). To obtain a larger amount of enzyme, the enzyme production in spinner culture was attempted. The time course of expression of the recombinant enzyme in spinner culture was monitored by enzyme activity, protein staining, and Western blotting with anti-rat methionine synthase antiserum. In the experiment shown in Fig. 1, Sf21 cells were infected with the recombinant virus at a multiplicity of infection of 0.8. The expression of functional apoenzyme reached maximum in 3 days postinfection in this spinner culture.
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Purity and Spectrum of the Purified Enzyme—The purity of the enzyme at each purification step was analyzed by SDS-PAGE (Fig. 2). The band with MW of 140,000 corresponds to rat methionine synthase (22). The summary of the purification procedures is shown in Table I. The recombinant rat methionine synthase was purified 71-fold in an overall yield of 8%. Specific activity of the purified enzyme was 1,980 milliunits/mg of protein, which is in good agreement with that (1,750 milliunits/mg of protein) of the purified rat liver enzyme (22). The purity of the enzyme thus obtained was established to be homogeneous, as judged by SDS-PAGE (Fig. 2, Mono-Q lane).

The electronic spectrum of the recombinant rat methionine synthase (purity, approximately 50%) is shown in Fig. 3. The spectrum observed resembled that of cob(II)alamin. This indicates that the highly purified enzyme underwent demethylation during purification, although the enzyme produced reconstituted holoenzyme with added MeCbl. It was also demonstrated that the spectrum changed to a methylcobalamin-like one upon incubation of the enzyme with Ti(III) citrate and AdoMet (Fig. 3), indicating reductive remethylation of the enzyme-cob(II)alamin complex.

Steady-state Kinetics and Requirements for a Reducing Agent and AdoMet—The affinities of the recombinant enzyme for substrates MeH<sub>4</sub>F and homocysteine were evaluated by the conventional steady-state kinetics under the standard assay conditions. The apparent $K_m$ values for MeH<sub>4</sub>F and homocysteine obtained from Lineweaver-Burk plots were 89 and 2.6 $\mu$M, respectively. These are in good agreement with those obtained with purified rat liver enzyme (75 and 1.7 $\mu$M, respectively) (22).

Because it has been established with the $E$. coli Cbl-depend-
methionine synthase reaction has remained unclear, although this moiety was suggested to be required for holoenzyme formation (12). Coenzyme activity of nucleotide moiety-modified analogs of MeCbl for methionine synthase was determined with crude extract by the anaerobic assay using Ti(III). As shown in Table II, MeCbi, an analog of MeCbl lacking the entire nucleotide moiety, was almost inactive as a coenzyme with extremely low activity (100 µM) of MeCbl in the absence of a reducing agent even when assayed with added MeCbl (data not shown). This suggested that a tight complex was not formed between the apoenzyme and MeCbi. In contrast, MeCbiP-Me, an analog of MeCbl lacking 5,6-dimethylbenzimidazole and D-ribose, showed low but not able to form the complex with apoenzyme. The enzyme activity was completely lost upon incubation at 37 °C for 5 min with a higher concentration (100 µM) of MeCbl in the absence of a reducing agent even when assayed with added MeCbl (data not shown). This suggested that a tight complex was not formed between the apoenzyme and MeCbi. In contrast, MeCbiP-Me, an analog of MeCbl lacking 5,6-dimethylbenzimidazole and D-ribose, showed low but significant coenzyme activity. The binding affinity of apoenzyme for this analog was much higher than that for MeCbl. Therefore, it can be concluded that the phosphodiester group of the nucleotide moiety plays an important role in the cobalamin binding to apoenzyme and thus for catalysis.

Because the bulkiness of the 5,6-dimethylbenzimidazole ligand was shown to be important for preventing diol dehydratase from suicide inactivation (15, 24), time course of the methionine synthase reaction with MeCbiP-Me as a coenzyme was compared by the anaerobic assay using Ti(III) with that with MeCbl. However, the methionine synthase reaction versus time curve with this analog was essentially linear within at least 15 min (data not shown). This indicates that the nucleotide moiety is necessary for the tight coenzyme binding by the apoenzyme but does not affect the rate of inactivation of methionine synthase during catalysis in the presence of Ti(III) and AdoMet.

**DISCUSSION**

Characterization of mammalian methionine synthase has been hampered by its low content in tissues. We obtained only 35 µg of homogeneous methionine synthase from 600 g of rat livers (22). The gene engineering techniques permitted us to get a recombinant protein in much higher quantity. We isolated the coding region of rat methionine synthase cDNA by reverse transcription-polymerase chain reaction (10). Production of recombinant methionine synthase in procaryotic cells was first attempted, because the primary structure of the rat enzyme is very similar to that of the E. coli enzyme (11). Although the expression of the functional enzyme was not successful, the inactive methionine synthase polypeptide was produced in E. coli cells using a T7 expression system. The antiserum against
The rat enzyme was raised using this polypeptide as antigen and used for detecting methionine synthase protein by Western blot analysis.

We then attempted heterologous expression of the recombinant enzyme in insect cells using a baculovirus expression system. As expected, the recombinant rat enzyme was obtained in a catalytically active form. The amount of recombinant rat methionine synthase purified from 500 ml of culture was almost comparable with that of the enzyme purified from approximately 2 kg of rat livers, indicating how efficiently the insect cells produce active mammalian enzyme. Specific activity as well as apparent $K_m$ values for substrates obtained with the recombinant enzyme were almost the same as those obtained with the rat liver enzyme (22).

Although the recombinant baculovirus was constructed using a single coding region of rat methionine synthase cDNA, the rat enzyme was raised using this polypeptide as antigen and used for detecting methionine synthase protein by Western blot analysis.

**Table II**

Coenzymic activity of MeCbl and MeCblP-Me for methionine synthase

| Corrinoid | Coenzyme activity | Apparent $K_m$ or $K_i$ |
|-----------|-------------------|------------------------|
|           | nmol/min/mg of protein | % | µM |
| MeCbl     | 33.0              | 100 | 0.34 ($K_m$) |
| MeCblP-Me | 4.7               | 14  | 2.5  ($K_m$) |
| MeCbl     | 1.3               | 4   | 35   ($K_i$) |

**Table III**

Specificity for upper axial ligands of Cbl in holoenzyme formation

| Cbl added | Holoenzyme activity | Ratio of holoenzyme activity to total activity |
|-----------|---------------------|---------------------------------------------|
|           | nmol/min/mg of protein | % |
| MeCbl     | 26.9                | 100 |
| aqCbl     | 3.5                 | 13  |
| CN-Cbl    | 0.1                 | 0.3 |

**Table IV**

Specificity for alkylcobalamins in holoenzyme formation with and without photolysis

| Cbl added | Photolysis | Holoenzyme activity | Ratio of holoenzyme activity to total activity |
|-----------|------------|---------------------|---------------------------------------------|
|           | nmol/min/mg of protein | % |
| MeCbl     | 26.2       | 100                |
| AdoCbl    | 0.0        | 0                  |
| +         | 0.7        | 3                  |
| EtCbl     | 0.1        | 0                  |
| +         | 26.2       | 100                |
| PrCbl     | 0.8        | 3                  |
| +         | 29.1       | 100                |

**Figure 4.** Time courses of the methionine synthase reaction with Ti(III) in the presence and absence of AdoMet. Crude extract was subjected to gel filtration on Sephadex G-25 and then incubated at 37 °C for 10 min with 10 µM MeCbl. The methionine synthase reaction was carried out with (●) and without (○) AdoMet under the anaerobic assay conditions using Ti(III) citrate, as described in the text.
two peaks of the enzyme activity were obtained upon Mono-Q column chromatography. These peaks of activity appeared in the same positions as those of the rat liver enzyme (22) upon elution from the Mono-Q column. Therefore, it is likely that the two peaks of enzyme activity observed upon purification of the enzyme from rat livers are derived from the single gene product. But we do not know at present how they were different.

The recombinant enzyme expressed in the insect cells grown without added Cbl was largely in the form of apoenzyme. This was very useful for studying the corrinoid specificity in catalysis and in holoenzyme formation. In general, resolution of holomethionine synthase into the apoenzyme accompanies a significant loss of activity (12). The apoenzyme was extremely unstable and therefore used without purification.

X-ray crystallographic analysis of the Cbl-binding domain of the \textit{E. coli} methionine synthase revealed that Cbl is bound to the enzyme with the nucleotide ligand displaced from the cobalt atom and accommodated in the binding pocket as an extended tail and with the imidazole group of a certain histidine residue of the protein ligated to the cobalt atom (5). Cobinamide was reported to be an inactive coenzyme for the \textit{E. coli} enzyme and ineffective in holoenzyme formation (12), suggesting the necessity of the nucleotide moiety for reconstituting holoenzyme. Our results reported here also showed that McCbl was essentially inactive as coenzyme for the recombinant rat enzyme and did not form a stable complex with apoenzyme. In contrast, Kolhouse et al. (13) reported that aquacobinamide is capable of activating human apoCbl fully at a lower concentration than that of aqCbl, when they are reduced with 2-mercaptoethanol. It would be reasonable to assume that the properties and the mechanism of action of the mammalian enzymes would be very similar to those of the bacterial enzyme, because both enzymes exhibited high similarity in the deduced amino acid sequences (7–11).

Ishida et al. (25) reported that McCbl analogs in which the ribose of the nucleotide moiety is replaced by a di- to penta-methylene group and a trimethylene analog containing imidazole instead of 5,6-dimethylbenzimidazole are partially or completely unable to activate human Cbl. The nucleotide moiety of Cbl is needed for the tight binding by its upper ligand. Alkylcobalamins with a small alkyl group must take place upon binding of Cbl to apoenzyme (5), it seems reasonable that the weaker coordination of the 5,6-dimethylbenzimidazole ligand to the cobalt atom favors the formation of complexes with alkylcobalamins. AdoCbl did not form a complex with apomethionine synthase. This is consistent with the result obtained with the \textit{E. coli} enzyme (26) and may be due to the bulkiness of its upper ligand, because the X-ray analysis of the Cbl-binding domain of the \textit{E. coli} enzyme showed the presence of a cap-like structure above the upper axial ligand (5).

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