Purification and Some Properties of Cobalamin-Dependent Methionine Synthase from Rat Liver

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Summary Cobalamin-dependent methionine synthase was purified from rat liver. The enzyme activity was separated into two peaks upon Mono-Q column chromatography. Peaks I and II of the enzyme, eluted in this order, were purified 18,000- and 44,000-fold in overall yields of 0.7 and 1.8%, respectively. Peak II methionine synthase, the major fraction, was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. The enzyme was a large monomeric protein with an apparent molecular weight of 143,000 Da. Interconversion of the enzyme between the two peaks was not observed during purification procedures. The enzyme required *S*-adenosylmethionine and a reducing system for activity. Apparent *K_m* values of the peak II enzyme for 5-methyltetrahydrofolate and homocysteine were 75 and 1.7 μ M, respectively.

Key Words cobalamin, vitamin B₁₂, methionine synthase, 5-methyltetrahydrofolate-homocysteine methyltransferase, rat liver, *S*-adenosylmethionine

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase [EC 2.1.1.13]) is one of the two mammalian enzymes known to be dependent on Cbl. The enzyme catalyzes the transmethylation from MeH₄F to L-homocysteine, forming L-methionine and H₄F. L-Methionine is used for protein synthesis or converted to AdoMet. AdoMet is a primarily important methylating agent in biological transmethylation reactions as well as a substrate for polyamine synthesis. H₄F serves as an essential cofactor in one-carbon unit metabolisms including amino

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; BPB, Bromophenol Blue; Cbl, cobalamin; DTT, dithiothreitol; OH-Cbl, hydroxocobalamin; MeCbl, methylcobalamin; MeH₄F, 5-methyltetrahydrofolate; SDS, sodium dodecyl sulfate; H₄F, tetrahydrofolate.

acid and nucleic acid metabolisms. Methionine synthase can thus be considered to play a pivotal role in these major biosynthetic pathways.

Insufficient methionine synthase activity appears to be associated with Cbl-deficiency diseases in human, although details of the mechanisms of diseases are not fully understood yet (1). The decrease of methionine synthase activity in several tissues has been shown with dietary Cbl-deficient rats (2,3) and fruit bats (4), and dietary cobalt-deficient sheep (5). Decreases in the activities of other methionine-metabolizing enzymes, such as AdoMet synthetase and cystathionine β -synthase, as well as in the level of AdoMet have also been observed in the liver of severely dietary Cbl-deficient rats (2,6).

In order to clarify the regulation of methionine synthase activity in mammals on a molecular level, we first attempted to isolate Cbl-dependent methionine synthase from rat liver. In this paper, we report the purification and some properties of methionine synthase from rat liver. Mammalian methionine synthase has so far been purified to apparent homogeneity from pig liver (7) and human placenta (8).

MATERIALS AND METHODS

Materials. Rat livers were obtained from Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan. AdoMet chloride and MeH₄F barium salts were purchased from Sigma, St. Louis, USA. Homocysteine was obtained from Aldrich, Milwaukee, USA. [methyl-¹⁴C]MeH₄F was a product of Amersham, Buckinghamshire, UK. DE-52 and Butyl- and DEAE-TOYOPEARL were purchased from Whatman, Maidstone, UK and Tosoh, Tokyo, Japan, respectively. Hydroxyapatite (fast-flow type) was purchased from Wako, Osaka, Japan. A FPLC system equipped with a Mono-Q 5/5 column (Pharmacia Biotech Inc., Uppsala, Sweden) or CHT-I (hydroxyapatite) column (Bio Rad, Hercules, USA) was used. OH-Cbl was prepared by photolysis of MeCbl. MeCbl was synthesized by the reaction of cob(I)alamin with methyl iodide (9).

Enzyme and protein assays. Methionine synthase activity was measured essentially as described by Frasca et al. (10). The reaction mixture contained 0.13 mM [¹⁴C]MeH₄F (specific activity 2,000 dpm/nmol), 0.8 mM homocysteine, 25 mM DTT, 0.1 mM AdoMet, 50 μ M OH-Cbl and the enzyme in 0.25 mL of 0.05 M potassium phosphate buffer (pH 7.5). After 10 min at 37°C, the reaction was terminated by quick chilling and dilution with two volumes of ice-cold water. The amount of methionine formed was determined using the Dowex 1 (Cl⁻) method described by Weissbach et al. (11). One unit of methionine synthase was defined as the amount of enzyme activity that catalyzes the formation of 1 μ mol of methionine/min at 37°C.

Protein concentration was determined by the method of Bradford (12) with bovine serum albumin as the standard.

Determination of the molecular weight of methionine synthase. The apparent

molecular weight of the native enzyme was estimated by electrophoresis on a polyacrylamide gradient gel (Multigel 2/15, Daiichi Pure Chemicals, Tokyo, Japan) under non-denaturing conditions (13). The relative molecular weight of the subunit was determined by electrophoresis on 12.5% polyacrylamide gel under denaturing conditions as described by Laemmli (14), except that bisacrylamide was used at a concentration of 0.5% that of acrylamide. Proteins were stained using a Silver Stain II kit (Wako, Osaka, Japan) according to the manufacturer's instructions.

RESULTS

Purification of methionine synthase from rat liver

All of the procedures were performed at 0–4°C. To minimize inactivation during purification, the procedures were done as quickly as possible.

Step 1: Preparation of crude extract from rat liver. About 100 g of rat livers were placed in 400 mL of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.05 M KCl and 1 mM phenylmethanesulfonyl fluoride and homogenized with a warring blender for 1 min at 10,000 rpm. The homogenate was centrifuged at $15,000 \times g$ for 1 h, and the supernatant was used as the crude extract.

Step 2: Batch chromatography on DE-52 (DEAE-cellulose). About 100 g of dry DE-52 was preliminarily equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) containing 0.05 M KCl. After filtration, the filter cake was added to the crude extract, and the suspension was gently stirred for 30 min. The resin was then filtered and washed with five volumes of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.05 M KCl. The enzyme was eluted with 450–500 mL of 0.3 M potassium phosphate buffer (pH 7.5) containing 0.3 M KCl.

Step 3: Ammonium sulfate precipitation. Finely powdered ammonium sulfate was added to the enzyme solution to a final concentration of 45% of saturation. After 20 min at 4°C, the suspension was centrifuged at $10,000 \times g$ for 10 min. The precipitate was re-dissolved in 200 mL of 0.01 M potassium phosphate buffer (pH 7.5) containing 0.5 M KCl.

Step 4: Hydroxyapatite column chromatography. The enzyme solution was loaded onto a hydroxyapatite column (3 × 6 cm) preliminarily equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) containing 0.5 M KCl. After washing the column with 250 mL of the same buffer, the enzyme was eluted with 150 mL of 0.15 M potassium phosphate buffer (pH 7.5).

Step 5: Butyl-TOYOPEARL column chromatography. Solid ammonium sulfate was added to the enzyme solution to a final concentration of 0.75 M, and the solution was immediately loaded onto a Butyl-TOYOPEARL column (1.5 × 5 cm) equilibrated with 0.15 M potassium phosphate buffer (pH 7.5) containing 0.75 M ammonium sulfate. The column was washed successively with 25 mL of the same buffer and then with 25 mL of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.75 M ammonium sulfate and 20% glycerol. The enzyme was eluted with 15 mL of 0.05 M

Tris-HCl buffer (pH 7.5) containing 0.55 M ammonium sulfate and 20% glycerol. The eluate was dialyzed overnight against 40 volumes of 0.05 M Tris-HCl buffer (pH 7.5).

Step 6: DEAE-TOYOPEARL column chromatography. The dialyzate was loaded onto a DEAE-TOYOPEARL column (1×3 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). After washing the column with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M KCl, the enzyme was eluted with 5 mL of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.2 M KCl.

Step 7: First Mono-Q column chromatography. The enzyme processed through step 6, from about 200 g of livers, was combined and loaded onto a Mono-Q column equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.2 M KCl. The FPLC system was operated at a flow rate of 0.5 mL/min and 1 mL-fractions were collected. The column was eluted with a 30-mL linear gradient from 0.2 to 0.35 M KCl in 0.02 M Tris-HCl buffer (pH 7.5). A typical elution profile is shown in Fig. 1A. Methionine synthase activity was eluted in two peaks at approximately 0.27 and 0.32 M KCl. The first and second peaks of enzyme activity, designated peaks I and II, respectively, were pooled separately.

Step 8: Second Mono-Q column chromatography. The enzyme processed through step 7, from 600 g of livers, was combined and dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) containing 0.1 M KCl. Dialyzed peaks I and II were re-chromatographed on a Mono-Q column under the same conditions as in step 7. The chromatograms obtained with peaks I and II are shown in Fig. 1B and C, respectively. Interconversion between peaks I and II was not observed.

Step 9: CHT-I (hydroxyapatite) column chromatography. Peaks I and II of the previous step were dialyzed overnight against 0.05 M potassium phosphate buffer (pH 7.5) containing 0.2 M KCl and applied to a CHT-I column (0.7×5.2 cm) equilibrated with the dialysis buffer. When peak I was eluted with a 30-mL linear gradient from 0.05 to 0.15 M potassium phosphate buffer (pH 7.5) containing 0.2 M KCl, enzyme activity appeared at ~0.1 M potassium phosphate buffer. When peak II of activity was applied, the column was washed with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.2 M KCl and then eluted with a 30-mL linear gradient from 0.1 to 0.2 M potassium phosphate buffer (pH 7.5) containing 0.2 M KCl. Peak II of activity appeared at approximately 0.15 M potassium phosphate buffer. Again, interconversion between peaks I and II was not observed during CHT-I column chromatography. The summary of the purification procedures for methionine synthase is shown in Table 1.

Purity and specific activity of the purified enzyme

SDS-polyacrylamide gel electrophoretic analysis of the enzyme at different stages of purification was performed (Fig. 2). Densitometric analysis of the stained gel indicated that the density of a band at 135,000 Da was essentially proportional to the amount of enzyme activity loaded to each lane (data not shown). Thus, it was evident that the band at 135,000 Da was progressively enriched, suggesting that

Fig. 1. Elution profiles of Mono-Q column chromatography. A. 1st Mono-Q chromatography. Two peaks of activity were named as peaks I and II according to the order of elution. B. Re-chromatography of peak I on Mono-Q. Peak fractions dialyzed were injected and eluted under the same conditions as in A. C. Re-chromatography of peak II on Mono-Q. Peak fractions dialyzed were injected and eluted under the same conditions as in A.

Table 1. Summary of the purification of cobalamin-dependent methionine synthase from rat liver.^a

Purification step	Total activity ^b (milliunits)	Total protein (mg)	Specific activity (milliunits/mg of protein)	Yield (%)	Purification (fold)
Crude extract	3,450	84,400	0.04	100	1
DE-52	1,530	11,100	0.14	44.3	3.5
(NH ₄) ₂ SO ₄	1,500	11,100	0.14	43.5	3.5
Hydroxyapatite	832	1,290	0.64	24.1	16.0
Butyl-TOYOPEARL	394	136	2.90	11.4	72.5
DEAE-TOYOPEARL	525	57.8	9.09	15.2	227
1st Mono-Q Peak I	84.1	5.49	15.3	2.4	383
2nd Mono-Q ^b	96.8	1.59	60.9	2.8	1,520
CHT-I ^b	25.3	0.035	723	0.7	18,100
1st Mono-Q Peak II	183	2.67	68.3	5.3	1,710
2nd Mono-Q ^b	125	0.796	157	3.6	3,930
CHT-I ^b	61.2	0.035	1,750	1.8	43,800

^a Purification from 600 g of rat livers.

^b Peaks I and II of the enzyme from the previous step were separately applied onto 2nd Mono-Q and CHT-I columns.

Fig. 2. SDS-polyacrylamide gel electrophoresis of fractions at each purification step. Samples boiled in the presence of 32 mM DTT and 1% (w/v) SDS were electrophoresed in 12.5% acrylamide gel and stained by silver staining as described in the text. Lane 1, crude extract from rat liver; lane 2, DE-52 eluate; lane 3, ammonium sulfate fraction; lane 4, hydroxyapatite eluate; lane 5, Butyl-TOYOPEARL eluate; lane 6, DEAE-TOYOPEARL eluate; lanes 7 and 10, 1st Mono-Q peaks I and II, respectively; lanes 8 and 11, 2nd Mono-Q peaks I and II, respectively; lanes 9 and 12, CHT-I peaks I and II, respectively. Molecular weight markers, Sigma MW-SDS-6H. The enzyme activity loaded was 0.01 milliunit in lanes 1 to 6, 0.08 milliunit in lanes 7 to 9, 0.3 milliunit in lanes 10 to 12. Arrowhead indicates the position of methionine synthase.

this band corresponds to methionine synthase. Peak II methionine synthase purified 44,000-fold was found to be homogeneous (Fig. 2, lane 12). The overall yield was 1.8% and the specific activity of the purified peak II enzyme was 1.8 units/mg of protein, which is comparable with the reported value for the purified enzyme from pig liver (13). The peak I enzyme was purified 18,000-fold with an overall yield of 0.7%. It showed a specific activity of 0.7 unit/mg of protein, but it was found to be still not homogenous. Densitometric analyses of the gels stained with quick-CBB (Wako) and by silver staining indicate that the purity of the peak I enzyme was approximately 44%. Therefore, the specific activity of a homogeneous peak I enzyme could be calculated to be ca. 1.6 units/mg of protein, which is comparable to that of the peak II enzyme.

Estimation of apparent molecular weight

The apparent molecular weight of the peak II enzyme purified to homogeneity was determined by non-denaturing polyacrylamide gel electrophoresis on a gradient gel (Multigel 2/15). The purified enzyme migrated as a single band, and its molecular weight obtained from Fig. 3 was 143,000 Da. The molecular weight of the subunit estimated by SDS-polyacrylamide gel electrophoresis was 135,000 Da (Fig. 2, lane 12). These results suggest strongly that methionine synthase from rat liver is a large monomeric protein.

Steady-state kinetics and requirement for AdoMet

The apparent K_m values of the peak II enzyme for MeH_4F and homocysteine were determined by conventional double reciprocal plots with a fixed concentration of the other substrate. Typical results are shown in Fig. 4. Apparent K_m values for MeH_4F and homocysteine were $75\ \mu\text{M}$ and $1.7\ \mu\text{M}$, respectively, although it is difficult to obtain an accurate K_m for homocysteine because of its very low value.

The requirement for AdoMet was examined since AdoMet is known to be

Fig. 3. Molecular weight determination of rat liver methionine synthase by non-denaturing polyacrylamide gel electrophoresis. Arrow indicates the migration distance of methionine synthase. Molecular weight markers: 1, thyroglobulin; 2, ferritin; 3, β -amylase; 4, alcohol dehydrogenase; 5, bovine serum albumin dimer.

Fig. 4. Lineweaver-Burk plots for MeH₄F and homocysteine with peak II methionine synthase. The means of duplicates or triplicates are shown. The enzyme in the final step of purification was used. The apparent K_m values for MeH₄F and homocysteine were obtained at fixed concentrations of homocysteine and MeH₄F of 0.8 mM and 0.13 mM, respectively. The conversion of substrate to product was less than 10% even at the lowest homocysteine concentration. Therefore, changes in the substrate concentrations during assay can be considered almost negligible. A. MeH₄F. B. Homocysteine.

absolutely required by *Escherichia coli* Cbl-dependent methionine synthase for reactivation through reductive remethylation of the inactivated holoenzyme (15). Like the pig liver enzyme (7), the rat liver enzyme also required AdoMet in the presence of DTT almost absolutely for activity, although AdoMet was still not saturated even at 0.1 mM (data not shown).

DISCUSSION

In this paper, we reported 44,000-fold purification of methionine synthase from rat liver. Purified peak II enzyme was homogeneous and showed specific activity of 1.8 units/mg of protein. This value was comparable with that of the purified enzyme from pig liver (7), more than 100-fold higher than that of the purified enzyme from human placenta (8), but six-fold lower than that of the purified *E. coli* enzyme (10). Since Cbl-dependent methionine synthase requires a reducing system and AdoMet for activation by reductive methylation of the

inactivated holoenzyme (15), such difference may be partly due to the difference of reducing systems and partly due to the concentration of AdoMet employed for the methionine synthase assays. An almost absolute requirement of rat liver methionine synthase for AdoMet was observed. This is consistent with the results obtained with pig liver enzyme (7). The apparent molecular weight of 143,000 Da for the native enzyme and relative molecular weight of 135,000 Da for the subunit suggest that methionine synthase from rat liver is a large monomeric protein. These values are similar to those obtained with the pig liver enzyme. The human placental enzyme, with a molecular weight of 160,000 Da, was reported to be a heterotrimer (8). However, upon Western blot analysis of partially purified enzyme from human placenta, a single band at 155,000 Da was shown to be cross-reactive with antibodies raised against the pig liver enzyme (16).

Methionine synthase activity was eluted in two peaks on anion-exchange and hydroxyapatite column chromatography, in accord with the results of Mellman et al. (17). In contrast to the pig liver enzyme (7), interconversion between them was not observed during Mono-Q and CHT-I column chromatography. These two forms of enzyme may be the products of limited proteolysis during purification or post-translational modification, such as phosphorylation (18), or the isoforms of rat liver methionine synthase. Their physiological significance remains to be elucidated.

Besides methionine synthase, there are three enzymes known to use homocysteine as a substrate: betaine homocysteine methyltransferase, cystathionine β -synthase, and S-adenosylhomocysteine hydrolase. The highest affinity of rat liver methionine synthase for homocysteine among the four enzymes suggests that this enzyme plays a major part in metabolizing homocysteine, since this amino acid usually exists in tissue at a very low concentration.

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REFERENCES

- 1) Beck WS. 1975. Metabolic features of cobalamin deficiency in man. In: Cobalamin Biochemistry and Pathophysiology (Babior BM, ed), p. 403-450. John Wiley and Sons, New York.
- 2) Doi T, Kawata T, Tadano N, Iijima T, Maekawa A. 1989. Effect of vitamin B₁₂-deficiency on the activity of hepatic cystathionine β -synthase in rats. *J Nutr Sci Vitaminol* 35: 101-110.
- 3) Kawata T, Takeda T, Morimoto F, Fujimoto F, Tanaka N, Yamada K, Wada M, Tadokoro T, Maekawa A. 1992. Effects of vitamin B₁₂-deficiency on testes tissue in rats. *J Nutr Sci Vitaminol* 38: 305-316.
- 4) Metz J, van der Westhuyzen J. 1987. The fruit bat as an experimental model of the neuropathy of cobalamin deficiency. *Comp Biochem Physiol* 88A: 171-177.
- 5) Kennedy DG, Blanchflower WJ, Scott JM, Kennedy S, Young PA. 1992. Cobalt-

- vitamin B-12 deficiency decreases methionine synthase activity and phospholipid methylation in sheep. *J Nutr* **122**: 1384–1390.
- 6) Doi T, Kawata T, Tadano N, Iijima T, Maekawa A. 1989. Effect of vitamin B₁₂ deficiency on S-adenosylmethionine metabolism in rats. *J Nutr Sci Vitaminol* **35**: 1–9.
 - 7) Chen Z, Crippen K, Gulati S, Banerjee R. 1994. Purification and kinetic mechanism of a mammalian methionine synthase from pig liver. *J Biol Chem* **269**: 27193–27197.
 - 8) Utley CS, Marcell PD, Allen RH, Antony AC, Kolhouse JF. 1985. Isolation and characterization of methionine synthase from human placenta. *J Biol Chem* **260**: 13656–13665.
 - 9) Smith EL, Mervyn L. 1963. Coenzyme B₁₂ and analogs synthesized from hydrido-cobalamin. *Biochem J* **86**: 2 p–3 p.
 - 10) Frasca V, Banerjee RV, Dunham WR, Sands RH, Matthews RG. 1988. Cobalamin-dependent methionine synthase from *Escherichia coli* B: Electron paramagnetic resonance spectra of inactive form and active methylated form of the enzyme. *Biochemistry* **27**: 8458–8465.
 - 11) Weissbach H, Peterkofsky A, Redfield BG, Dickerman H. 1963. Studies on the terminal reaction in the biosynthesis of methionine. *J Biol Chem* **238**: 3318–3324.
 - 12) Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.
 - 13) Lambin P, Fine JM. 1979. Molecular weight estimation of proteins by electrophoresis in linear polyacrylamide gradient gels in the absence of denaturing agents. *Anal Biochem* **98**: 160–168.
 - 14) Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
 - 15) Taylor RT, Weissbach H. 1969. *Escherichia coli* B N⁵-methyltetrahydrofolate-homocysteine cobalamin methyltransferase: Activation with S-adenosyl-L-methionine and the mechanism for methyl group transfer. *Arch Biochem Biophys* **129**: 745–766.
 - 16) Chen Z, Chakraborty S, Banerjee R. 1995. Demonstration that mammalian methionine synthases are predominantly cobalamin-loaded. *J Biol Chem* **270**: 19246–19249.
 - 17) Mellman IS, Youngdahl-Turner P, Willard HF, Rosenberg LE. 1977. Intracellular binding of radioactive hydroxocobalamin to cobalamin-dependent apoenzymes in rat liver. *Proc Natl Acad Sci USA* **74**: 916–920.
 - 18) Kenyon SH, Ast T, Gibbons WA. 1995. In vitro phosphorylation of vitamin B₁₂ dependent methionine synthase by protein kinase A. *Biochem Soc Trans* **23**: 335S.