Isolation and Characterization of Methionine Synthetase from Human Placenta*

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The covalamin-dependent enzyme, methionine synthetase, has been purified approximately 1000-fold to apparent homogeneity from human placenta with a 19% recovery.

The final two steps of the purification utilized two different affinity columns. The first was a N5-methyltetrahydrofolate-cystamine-agarose column, and the second was a S-adenosylhomocysteine-agarose column. The enzyme was eluted from the first affinity column by buffer containing reduced sodium chloride. However, analysis by high performance liquid chromatography using a molecular weight sizing column demonstrated a single peak of protein with an apparent molecular weight of 160,000 were obtained by gel filtration and that holomethionine synthetase contained 1 mol of cobalamin/mol of protein. Furthermore, analysis by high performance liquid chromatography using a molecular weight sizing column demonstrated a single peak of protein with a corresponding cobalamin peak. This single peak of protein was progressively converted to a second protein peak that was enzymatically inactive, and this conversion was associated with a directly proportional loss of enzyme activity and cobalamin from the first peak.

Methionine synthetase appeared to have a molecular weight of 160,000 on unreduced sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and subunits of M0, 90,000, 45,000, and 35,000 on reduced sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis.

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Methionine synthetase (tetrahydropteroylglutamate; L-homocysteine-S-methyltransferase, EC 2.1.1.13) is one of the two mammalian enzymes known to depend on covalamin (Cbl) in coenzyme form (1). Studies of bacterial Met synthetase (2) indicated that both methyl-Cbl (Me-Cbl) and Cob(II)alamin were present bound to the enzyme, and a partially purified preparation of porcine kidney Met synthetase appeared to contain Cbl (3).

Most of the information on the function of Met synthetase comes from studies of purified Cbl-dependent bacterial Met synthetase. Met synthetase was shown (2) to catalyze the transfer of methyl groups from Me-Cbl to homocysteine to form methionine. These studies also suggested (2, 4) that the methyl group of N5-methyltetrahydrofolate (Me-Hfolate) was responsible for remethylating the Cbl following transfer of the methyl group to homocysteine while the N5-Me-Hfolate was converted to tetrahydrofolate in this reaction. However, the reaction also catalyzed the conversion of S-adenosylmethionine to S-adenosylhomocysteine (AdoHcy), and S-adenosylmethionine was suggested to occasionally serve as a methyl donor for remethylation of Cbl in this reaction (1).

Insufficient Met synthetase activity appears to be associated with a characteristic megaloblastic anemia that is unique to humans as opposed to other mammals (1). Deficiency of either folate (the substrate) or Cbl (the coenzyme) results in a morphologically indistinguishable megaloblastic anemia (1). Total Met synthetase (holo-Met synthetase plus apo-Met synthetase) activity was decreased in bone marrow cells of individuals deficient in Cbl (5), and the proportion of apo-Met synthetase activity was increased (6). Another study (7) demonstrated that the total Met synthetase activity was decreased in Cbl-deficient patients and that total Met synthetase activity increased to the lower limits of normal 72 h following Cbl replacement. These results suggest that Cbl not only influenced the proportion of holo-Met synthetase present, but that Cbl also influenced the quantity of total Met synthetase enzyme protein present.

In addition to acquired Met synthetase deficiency, genetic defects have been described that involve a decrease in the intracellular synthesis of Me-Cbl with an associated decrease in Met synthetase activity (8). Phenotypically, patients with these genetic defects have megaloblastic anemia, hypome-thioninemia, and homocystinuria (8). Fibroblasts from these patients contained markedly decreased Met synthetase activity that could be stimulated to approximately 50% of control activity by incubation with a variety of forms of Cbl (9).

The complexity of the Met synthetase enzyme reaction as well as the apparently unique function of this enzyme in humans prompted us to purify and characterize Met synthetase from human placenta.

EXPERIMENTAL PROCEDURES

Materials

N5-[methyl-14C]Tetrahydrofolic acid (barium salt, 56 mCi/nmol) and CN-[57Co]Cbl (300 μCi/nmol) were purchased from Amersham/
Purification of Human Methionine Synthetase

Searle, Arlington Heights, IL. dl-N-5-methyltetrahydrofolic acid (barium salt), methylmalonyl-CoA, bovine serum albumin (BSA), l-homocysteine thiolactone, l-methionine, CN-Cl, OH-Cl, Me-Cbl, S-adenosylmethionine (iodide form), Sephadex G-200, 2-mercaptoethanol, cystamine di-HCl, 1-ethyl-3-(3-dimethylamino- propyl)carbodiimide, PAD, trypsin (bovine pancreas), pepsin (porcine stomach), and phenanthroline sulfate were obtained from Sigma.

DE52 celluose was obtained from Whatman. Affi-Gel 202 was obtained from Bio-Rad. AdoHcy agaroase was obtained from Bethesda Research Laboratories. Ammonium sulfate (ultrapure) was obtained from Sigma. DEAE-cellulose was obtained from Whatman. Affi-Gel 202 was obtained from Bio-Rad. AdoHcy agaroase was obtained from Bethesda Research Laboratories. Ammonium sulfate (ultrapure) was obtained from Sigma.

Methods of Analysis

Enzyme Assays

Methionine synthetase (Met synthetase) was routinely assayed by a previously described method (10) except the final concentration of $N\textsubscript{5}$-methyltetrahydrofolic acid was 250 $\mu$M. Anaerobic assays for Met synthetase were performed as described (3, 22) except the incubation time at 30 °C was 11 min. An enzyme unit (U) was defined as 1 nmol of substrate converted per min, unless otherwise indicated. Holo-Met synthetase was taken to be the enzyme activity present in the absence of added Cbl. Total Met synthetase activity was determined in a duplicate assay that contained 40 $\mu$M Cbl. Apo-Met synthetase activity was taken to be the difference between holo-Met synthetase activity and total Met synthetase activity. Some measurements were determined in an anaerobic assay utilizing Cob(II)alamin without the 2-mercaptoethanol-FADH$_2$-reducing system of the routine method of assay. Anaerobic assays were performed under argon (99.9995%).

Synthesis of Cob(II)alamin

Cob(II)alamin was prepared from 4 mM OH-Cbl by incubation at 25 °C for 1 h. 2-OH-Cbl (the AdoHcy-agaroase eluate) was applied directly to the top of the gradient and centrifuged at 48,000 rpm for 21 h in a SW 50.1 rotor in a Beckman L5-65 centrifuge at 4 °C. Simultaneous gradients containing $^{14}$C-radiolabeled marker proteins (17) were also run. Ten drop fractions were collected from the bottom of the gel. Slab gels were dried using a Bio-Rad (model 224) gel slab dryer. The following were utilized as standards for determination of $M_2$: IgG, 158,000; transferrin, 80,000; bovine serum albumin, 67,000; and ovalbumin, 45,000.

Succrose Gradient Ultracentrifugation

Linear 5-25% sucrose gradients were prepared in 0.01 M KPO$_4$, pH 7.5. Continuous sucrose gradients were manually prepared by successively layering 0.9 ml of 25, 20, 15, 10, and 5% sucrose in polyallomer tubes and allowing equilibration to occur for 3 h at 22 °C followed by a 16-h incubation at 4 °C. The purified Met synthetase enzyme (peak of Cob(II)alamin) was applied directly to the top of the gradient and centrifuged at 48,000 rpm for 21 h in a SW 50.1 rotor in a Beckman L5-65 centrifuge at 4 °C. Simultaneous gradients containing $^{14}$C-radiolabeled marker proteins (17) were also run. Ten drop fractions were collected from the bottom of the tube using a Beckman fraction collector system. One ml of H$_2$O and 20 ml of scintillation fluid were added to the $^{14}$C-radiolabeled marker proteins. Each was counted for radioactivity in an Isocap scintillation counting system for $^{14}$C. The fractions from the purified Met synthetase enzyme sample were assayed for Met synthetase activity and protein.

Amino Acid Analysis

Purified Met synthetase was dialyzed against 1000 volumes of H$_2$O with three changes of dialysate and assayed for protein and Cbl. Following the addition of 50 nmol of norleucine as an internal standard, the sample was taken to dryness in a vacuum centrifuge. The sample was then dissolved in 6 $N$ HCl followed by heating at 105 °C for 24 h. The sample was then taken to dryness and the amino acid analysis was performed by C. H. W. Hirs, Department of Biochemistry, University of Colorado Health Sciences Center.

High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a Waters model 720 system controller, a model 730 data module, two model 6000A pumps, a model USK injector, and a model 440 dual channel absorbance detector with fixed and variable wavelengths. Detector wavelengths were 280 and 365 nm set at 0.05 and 0.01 absorbance units at full scale, respectively. The column was a Varian Micropak TSK-G3000SW 7.5 x 500 mm with a Micropak TSK-GSWP 7.5 x 75-mm guard column. Prior to the injection, samples were dialyzed for 5 h at 4 °C against

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Cbl measurements were performed utilizing either intrinsic factor or R-type protein radiodilution assays as previously described (10). A Varian Micropak TSK-G3000 SW 7.5 x 300-mm column with a Micropak TSK-GSWP 7.5 x 75-mm guard column was obtained from Varian Instrument Group, Palo Alto, CA.

Conversion of Apo-Met Synthetase to Holo-Met Synthetase

Purified Met synthetase (2.0 nmol) was incubated under argon with 0.01 M KPO$_4$, pH 7.5, containing 40 nmol of $^{14}$C-Co(II)alamin (0.1 $\mu$Cl in a final volume of 1 ml at 4 or 37 °C. After 40 min the solutions were placed in 10-mm dialysis tubing with a $M_2$, 12,000 cutoff (Spectrator, Spectrum Medical Industries, Los Angeles, CA). The samples were dialyzed at 4 °C in 4000 volumes of 0.01 M KPO$_4$, pH 7.5, for 24 h. Radioactivity in controls that contained no Met synthetase was subtracted from the samples that contained Met synthetase. Protein was assayed before and after dialysis to correct for recovery.

Gel Filtration

Gel filtration was performed on a 2.5 x 30-cm column of Sephadex G-200 at 4 °C. The column was equilibrated and eluted with 0.01 M pyridine-acetic acid, pH 7.4, at a flow rate of 20 ml/h. Three ml fractions were collected and blue dextran 2000 was utilized to determine the void volume. The column was calibrated with pure rabbit IgG, bovine serum albumin, ovalbumin, and chymotrypsinogen. Following the assay for enzyme activity, each fraction was taken to dryness in a Savant vacuum centrifuge prior to assays for protein and cobalamin.

Polyacrylamide Disc Gel Electrophoresis

Protein solutions were subjected to disc gel electrophoresis at pH 8.6 in Tris-glycine buffer as previously described (15). The electrophoresis was performed at 4 °C, and duplicate gels were either stained for protein with Coomassie Blue, stained for carbohydrate with periodic acid-Schiff, or sliced into 2-mm sections, eluted in 0.3 ml of 0.01 M KPO$_4$, pH 7.5, containing 0.15 M NaCl, and assayed for Met synthetase activity and Cbl content. Gels were also run with 7% thiglycollate in the sample and in the running buffer.

Analytical DEAE-cellulose Column

A 2.0 x 8-cm DEAE-cellulose column was equilibrated in 3.02 M sodium phosphate, pH 7.0. One hundred ml of centrifuged crude placental homogenate was applied. After washing with five column volumes of 0.02 M sodium phosphate, pH 7.0, a linear gradient of sodium chloride was initiated. The mixer bottle contained 125 ml of 0.02 M sodium phosphate, pH 7.0, while the reservoir bottle contained 125 ml of 0.02 M sodium phosphate, pH 7.0, with 0.4 M sodium chloride. Two-ml fractions were collected and assayed for total enzyme activity, and separate aliquots of the fractions were assayed for cobalamin by the radioisotope dilution assay (10).

Sodium Dodecyl Sulfate-Polyacrylamide Slab Gel Electrophoresis

Protein was dialyzed at 4 °C against 4000 volumes of H$_2$O for 18 h and dissolved in 1% SDS with and without 1% 2-mercaptoethanol. The samples were immediately boiled for 5 min. Staining and detaining procedures were performed as described by Laemmli (16). Five per cent polyacrylamide slab gels, 1.5 mm thick, were made on a 311-nm peak of Cob(I1)alamin (14). The final chro-
4000 volumes of 0.01 M KPO₄, pH 7.5, and then filtered through a Millipore HA 0.45-µm filter.

Cbl Determination

Solutions of crystalline OH-Cbl were dissolved in 0.01 M KPO₄, pH 7.5, and assayed by measuring absorbance at 278 nm. The molar extinction coefficient used to determine the concentration of OH-Cbl was Eₐ₅₀ = 13,100 (14). Dilutions of this stock solution of OH-Cbl were used to prepare standards for Cbl assays. In addition, various quantities (ranging from 25–2000 ng) of OH-Cbl were injected into the HPLC system to determine the peak areas given at 365 nm.

Absorption spectra were obtained on a Gilford 240 spectrophotometer with a wavelength-scanning attachment. Full scale absorbance was set at 0.290, and the scan rate was 120 nm/min.

Protein Determination

Protein was determined by the method of Lowry et al. (18) using BSA as a standard.

X-ray Fluorescence

X-ray fluorescence of the purified Met synthetase was performed as previously described (15).

Atomic Absorption Spectroscopy

Atomic absorption spectroscopy was performed by Coors Spectrochemical Laboratory, Golden, CO.

Cbl Release by Proteolysis

Met synthetase (50 µg) was incubated at 37 °C in HCl, pH 2.0, with 500 µg of pepsin in a final volume of 0.5 ml. After 30 min the pH was increased to 7.5 with 0.1 M NaOH, and the solution was adjusted to 0.1 M KPO₄, pH 7.5, and 0.15 M NaCl. Five hundred µg of trypsin was added. After incubation for 1 h at 37 °C the sample was boiled for 10 min and assayed for Cbl. Controls were performed with Met synthetase that contained no proteolytic enzymes and with a solution of OH-Cbl that contained proteolytic enzymes but no Met synthetase.

Purification of Human Methionine Synthetase

All procedures during purification were performed at 4 °C unless otherwise stated.

Step 1: Preparation of Placental Homogenate

Human full term placentas were obtained from healthy patients following vaginal delivery. The placentas were immediately immersed in an ice bath until used. The umbilical cord and membranes were removed. Each placenta was extensively washed with cold tap water to remove blood and blood clots. Small random samples of each placenta were combined, weighed, and frozen for 120 min at −70 °C. The remainder of each placenta was weighed and frozen for 120 min at −70 °C. The random samples were thawed and homogenized at 4 °C in 2.5 volumes of 0.01 M KPO₄, pH 7.5, containing 0.05 M NaCl. Homogenization was achieved with five 1-min bursts in a Waring blender. The homogenate was centrifuged at 40,000 × g for 30 min at 4 °C. The supernatant was decanted and used as the source of Met synthetase. If enzyme assays indicated levels of total activity greater than 0.0014 EU/g, wet weight (60% of all placentas), the main portion of the placenta was thawed and processed in the manner described above. The purification steps which follow were done on 1.1 kg of placental tissue prepared as stated above.

Step 2: Ammonium Sulfate Precipitation

Solid ammonium sulfate, 18 g/100 ml, was added to the supernatant (total volume of 3.6 liters) from the previous step. The ammonium sulfate was added gradually while the supernatant was constantly stirred on a magnetic stirrer. After 15 min of thorough stirring, the sample was centrifuged at 30,000 × g for 30 min. The pellet was discarded, and additional ammonium sulfate, 10.8 g/100 ml, was added to the supernatant which was stirred and centrifuged as above. The pellet was resuspended in a minimal volume of approximately 300 ml of 0.01 M KPO₄, pH 7.5, containing 0.05 M NaCl and dialyzed against 4 liters of the same buffer with changes at 2 and 16 h. The enzyme was then placed in 4 liters of 0.02 M NaPO₄, pH 7.0, and dialyzed an additional 3 h.

Step 3: DEAE-cellulose Chromatography

DEAE-cellulose was prepared by removing the fines, and final equilibration was made in 0.02 M NaPO₄, pH 7. The final dialyzed sample from the ammonium sulfate precipitation step was applied to a 2.5 × 45-cm column at a flow rate of 160–200 ml/h. The column was washed with 1 liter of 0.02 M NaPO₄, pH 7.0. The enzyme was eluted utilizing a NaCl gradient of 1 liter of 0.02 M NaPO₄, pH 7.0, in the mixing chamber and 1 liter of 0.02 M NaPO₄, pH 7.0, containing 0.4 M NaCl in the reservoir chamber. Fractions of 15 ml were collected and assayed for Met synthetase enzyme activity. The peak fractions (approximately 50% through the gradient), containing 70% of the total eluted activity, were combined, and a 50% ammonium sulfate precipitation was done. The ammonium sulfate pellet was resuspended in a volume of 70 ml of 0.01 M KPO₄, pH 7.5, and dialyzed against 4 liters of the same buffer with changes of the dialysate at 2, 16, and 18 h. The final dialyzed supernatant was collected and used as a source of enzyme for Step 4.

Step 4: N⁵-Methyltetrahydrofolate-Cystamine-Agarose Affinity Chromatography

Preparation of Affinity Gel—20 ml of Affi-Gel 202 was sequentially washed utilizing a fritted glass funnel (under vacuum) with 1 liter of H₂O, 1 liter of 1 M NaCl, and 1 liter of H₂O. To the moist cake approximately 10 ml of H₂O was added. To this solution cystamine di-HCl was added to a final concentration of 0.1 M, and the pH was adjusted to 5.0 using 1 N HCl. Solid 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was dissolved in H₂O at 100 mg/ml concentration. This solution was added in five equal parts to the above solution of cystamine and agargose over a 5-min period. The pH was adjusted to 5.0, and the final volume was made to 40 ml with H₂O. The solution was mixed on a tube rotator for 18 h at 22 °C.

The Affi-Gel 202-cystamine was washed using a fritted glass funnel with 2 liters of H₂O, 1 liter of 1 M NaCl, and 2 liters of H₂O. To the moist cake 10 ml of deoxygenated H₂O was added followed by solid N⁵-Me-H₄-folate to a final concentration of 0.1 M, and the pH was adjusted to 5.5 with 1 N HCl. To this solution was added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as described above. The final pH was adjusted to 5.5, and the final volume was made 40 ml with deoxygenated H₂O. Nitrogen gas was bubbled into the preparation for 10 min, and the preparation was kept dark at 22 °C on a tube rotator for 18 h.

Use of Affinity Gel—Immediately prior to application of Met synthetase obtained from Step 3, the affinity column was washed sequentially with 2 liters of H₂O, 1 liter of 1 M NaCl, and 2 liters of H₂O in a fritted glass funnel under vacuum. The Met synthetase in a volume of 70 ml was applied to a 15-ml column of N⁵-Me-H₄-folate-cystamine-agarose at a flow rate of 1 ml/min at 4 °C. Following application of the enzyme the column was washed with 300 ml of 0.01 M KPO₄, pH 7.5, followed by 300 ml of 0.01 M KPO₄, pH 7.5, containing 1 M NaCl. The Met synthetase was eluted by adding 15 ml of 0.01 M KPO₄, pH 7.5, containing 0.1 M 2-mercaptoethanol and 1 M NaCl. The column was allowed to stand for 18 h at 4 °C after which the enzyme was eluted at a flow rate of 1 ml/min. Additional Met synthetase activity was eluted with 30 ml of the same elution buffer at 22 °C. These eluates were combined and dialyzed against 4 liters of 0.01 M KPO₄, pH 7.5, with a change of dialysate at 16 and 18 h.

Step 5: AdoHcy-Agarose Affinity Column Chromatography

The dialyzed eluates (approximately 50-ml volume) from Step 4 were applied (at a flow rate of 1 ml/min) to a 15-ml AdoHcy-agarose column which had been preswelled with 100 ml of deionized H₂O followed by 100 ml of 0.01 M KPO₄, pH 7.5. After application of the sample, the column was washed with 150 ml of 0.01 M KPO₄, pH 7.5. The column was eluted at a flow rate of 1 ml/min at 4 °C with 30 ml of 0.01 M KPO₄, pH 7.5, containing 0.1 M NaCl followed by 15 ml of 0.01 M KPO₄, pH 7.5, containing 0.2 M NaCl followed by 15 ml of 0.01 M KPO₄, pH 7.5, containing 0.5 M NaCl and finally by 15 ml of 0.01 M KPO₄, pH 7.5, containing 1.0 M NaCl. Each salt concentration eluate was collected separately and assayed for protein and enzyme activity. The 0.5 and 1.0 M NaCl eluates contained 70% of the enzyme activity and were pooled and used as the source of purified Met synthetase.

To test the effect of a neutral protease inhibitor, a smaller (0.3 kg) placental preparation was used. Phenylmethylsulfonyl fluoride (1...
mM) was added to the homogenization buffer and all subsequent buffers.

RESULTS

Stimulation of Met Synthetase Activity by Cbl—Crude placental Met synthetase was consistently stimulated by addition of CN-Cbl to the enzyme assay. Total activity in the presence of 40 μM CN-Cbl minus activity in the absence of CN-Cbl (holo-Met synthetase) was considered apo-Met synthetase. The percent of holo-Met synthetase as well as total Met synthetase activity varied widely among different placentas. The total Met synthetase activity varied from 0.0004–0.0028 EU/g, wet weight, while the percentage of holo-Met synthetase varied from approximately 10–40%. Only placentas with greater than 0.0014 EU of total Met synthetase activity were utilized, and the crude homogenate that was utilized for the purification shown in Table I contained 24% holo-Met synthetase.

Intracellular Cbl-binding Proteins—Fig. 1 demonstrates that Met synthetase can be distinguished from the other known mammalian Cbl-dependent enzyme, methylmalonyl-CoA mutase (EC 5.4.99.2), by anion exchange chromatography utilizing a linear NaCl gradient elution. The Met synthetase eluted at a NaCl concentration of 0.23 M. Assay of Cbl in these peaks of methylmalonyl-CoA mutase and Met synthetase from human placenta indicated that greater than 90% of the bound Cbl was present bound to Met synthetase while the remainder was present bound to methylmalonyl-CoA mutase.

Not shown in Fig. 1 was the fact that 39% of the total Cbl measured in the crude placental homogenate did not bind to the column and was presumed to be free Cbl as has been observed in the past with animal tissues (12).

Stability of Met Synthetase—We purified the enzyme from frozen placentas over a 5-day period. Over this period, no activity was lost from the crude placental homogenate stored at 4°C. Once the enzyme was purified, activity was lost slowly (20%) up to 2 weeks at 4°C but more rapidly if the enzyme was frozen.

Purification of Methionine Synthetase—Table I contains the scheme utilized for purification of Met synthetase. The overall recovery of enzyme activity was 19% with approximately a 933-fold purification. The recovery of Met synthetase to the N5-Me-H4folate-cystamine-agarose column was virtually 100% but only approximately 70% of the enzyme applied bound to the column. The 30% of Met synthetase that did not bind to this column would not bind to another identical affinity column suggesting that approximately 30% of the Met synthetase from the DEAE column was in a form that would not bind to N5-Me-H4folate. The nature of this enzyme is unknown although failure to bind may be a reflection of endogenous N5-Me-H4folate occupying the binding site.

We were able to obtain 60% binding of Met synthetase to a N5-Me-H4folate-Sepharose column, but the recovery of Met synthetase from this column was less than 1% despite multiple attempts to elute the Met synthetase with various buffers with and without free N5-Me-H4folate. The interposition of the cystamine molecule allowed us to reverse the binding to this column by buffers containing 2-mercaptoethanol. In addition, the binding to the N5-Me-H4folate-cystamine-agarose column was specific for the folate form utilized. Replacement of the N5-Me-H4folate with N5-formyltetrahydrofolate or pteroylmonoglutamate resulted in less than 5% binding of Met synthetase.

Met synthetase was eluted from the AdoHcy-agarose column by increasing the NaCl concentration from 0.2–1.0 M. The 0.5 M NaCl elution consistently contained the most enzyme activity. However, a significant amount of activity was also eluted with 1.0 M NaCl. Since these eluates contained 70% of the total eluted enzyme, they were pooled and utilized as a source of purified Met synthetase.

HPLC—Since Mr sizing was not utilized as a step in the purification, we analyzed the purity of the final Met synthetase preparation by HPLC utilizing a Mr sizing column. Prior to studying the enzyme by HPLC, the system was calibrated.

![Graph](image_url)
by using known quantities of BSA and OH-Cbl. As expected, less than 5% of the injected BSA was detected in the A_{380} channel as compared to the A_{386} channel while as little as 1 μg of injected BSA could be easily detected in the A_{386} channel. As little as 50 ng of OH-Cbl could be detected in the A_{386} channel and the A_{386}/A_{380} ratio for OH-Cbl was 0.2 in this system. Thus, this system was very sensitive for the detection of protein and was also highly sensitive for the detection of OH-Cbl.

The results of studies of purified Met synthetase by HPLC are shown in Fig. 2. The results in Fig. 2 were obtained from a 1-ml solution of purified Met synthetase that was dialyzed against 1 liter of 0.01 M KPO_4, pH 7.5, for 3 h, and 50 μg were injected onto the column. This dialysis was important to obtain sharp A_{386} and A_{380} peaks presumably as a result of removal of NaCl. A single large peak of A_{386} and A_{380} at approximately 11 min retention time was observed. The results shown in Fig. 2, B and C, indicated that manipulations that resulted in loss of enzyme activity resulted in a proportional loss of protein and Cbl (A_{380}) in the peak with 11 min retention time. The inactivated enzyme appeared at 25 min retention time. Cbl (A_{380}) either remained bound to the protein or appeared at 28 min (Fig. 2C) near to the retention time at which free Cbl eluted. The sample shown in Fig. 2B was obtained by drying 1 ml of a solution of purified Met synthetase in 0.01 M KPO_4, pH 7.5, 0.15 M NaCl to a volume of less than 0.005 ml over 4 h at 22 °C. The sample was then reconstituted in 1 ml of H_2O, dialyzed against 1 liter of 0.01 M KPO_4, pH 7.5, for 3 h, and 50 μg were injected onto the column. Preinjection measurement of protein and Cbl gave results similar to the sample utilized in Fig. 2A, although the enzyme activity was reduced to 51% in this sample. The results shown in Fig. 2B indicated that approximately 50% of the 11-min peak was lost and was distributed between a 12-min peak (shoulder on first peak), followed by a broader intermediate retention time peak and finally a large peak with a retention time of 25 min. This latter peak also appeared to contain bound Cbl based on the presence of a peak in A_{386} channel. Prolonged drying of Met synthetase resulted in complete loss of enzyme activity and virtually complete disappearance of the 11-min retention time peak (Fig. 2C). The mechanism of breakdown of the protein following desiccation is unknown, and we cannot rule out proteolysis or degradation by other processes.

It was not possible to determine the contribution of Cbl to the A_{386} peak since the extinction coefficient for Cbl likely changed on binding to protein (19), and the form(s) of Cbl bound to the purified Met synthetase was unknown. That at least a portion of the A_{386} peak was due to Cbl was suggested since assayable Cbl was found in only those fractions with an A_{386} peak, and all A_{386} peaks contained nanogram quantities of assayable Cbl proportionate to the peak areas.

Gel Filtration Studies—To further analyze the purified Met synthetase, we applied the enzyme to a gel filtration column (Fig. 3) and assayed fractions for enzyme activity, protein and Cbl. All the enzyme activity, protein, and Cbl were high M_r (peak at 160,000), and no detectable protein or Cbl were present in fractions that did not contain Met synthetase activity. The specific activity of Met synthetase/unit of either protein or Cbl was virtually identical for each fraction. Similar studies were performed with purified Met synthetase on sucrose density gradients (data not shown). In these studies Met synthetase migrated with IgG (M_r = 158,000), and a single peak of protein and Met synthetase activity was obtained.

Stoichiometry of Cbl Binding to Methionine Synthetase—Table II contains data indicating that 90% of the Met synthetase was functionally and structurally apo-Met synthetase with regard to both stimulation by Cob(I1)alamin and to bound Cbl measured by atomic absorption. When the Met synthetase was incubated with Cob(I1)alamin at 37 °C (anaerobically) for 40 min, total residual Met synthetase activity was reduced by 55%, and 0.5 mol of Cob(I1)alamin was bound per mol of Met synthetase. In addition, the residual Met synthetase activity was not further stimulated on addition of Cob(I1)alamin to the assay, suggesting that the residual Met synthetase activity was all in the form of holo-Met synthetase.

When Cob(I1)alamin was incubated with Met synthetase at
4 °C (anaerobically) the residual Met synthetase activity was stable, the Met synthetase appeared to be all holo-Met synthetase, and 1 mol of Cob(I1)alamin/mol of Met synthetase was bound. The fact that 55% of enzyme activity was lost at 37 °C and the Cbl bound was reduced by 50% suggested that Met synthetase that lost activity would not bind Cbl. In addition, the fact that 1 mol of Cob(I1)alamin was bound per mol of enzyme was compatible with the fact that most (90%) of the purified enzyme was apo-Met synthetase with respect to Cbl. When identical studies were performed with CN-Cbl, no binding or functional conversion to holoenzyme was observed following dialysis. These observations not only suggested that CN-Cbl did not bind to the enzyme but also that after Cob(I1)alamin was bound to Met synthetase, the complex was stable in the presence of oxygen.

Polyacrylamide Gel Electrophoresis—Application of purified Met synthetase to native polyacrylamide gel electrophoresis resulted in loss of all enzyme activity and the appearance of multiple protein-staining bands (not shown) despite prerunning of the polyacrylamide gels or inclusion of 7 mM thioglycollate in the running buffer. Met synthetase purified in the presence of 1 mM phenylmethylsulfonyl fluoride to inhibit placental proteases gave a virtually identical pattern.

We also converted apo-Met synthetase to holo-Met synthetase with [57Co]Cob(I1)alamin. After running a gel using [57Co] Cob(I1)alamin-Met synthetase, gel slices were directly eluted and assayed for Cbl. These results were interesting since a single peak of [57Co]cobalamin and enzyme activity have been obtained using Met synthetase from human fibroblasts (23, 24). However, it was possible to obtain this data only if the intact fibroblasts were incubated with Cbl, since only holo-enzyme activity could be detected on the gel (24).

Fig. 4 contains the results of purified human Met synthetase run on SDS-slab gel electrophoresis. A major band (Fig. 4A) with a M, of 160,000 was observed on the unreduced gel. Two additional bands at molecular weights of 80,000 and 70,000 were also observed. Studies of Met synthetase purified in the presence of phenylmethylsulfonyl fluoride gave a similar gel profile. Reduction (Fig. 4B) of Met synthetase resulted in disappearance of the M, 160,000 band and the appearance of bands at M, 90,000, 45,000, and 35,000. No M, 70,000 or 80,000 bands were apparent on the reduced gel.

Spectrophotometry of Purified Methionine Synthetase—Fig. 5 contains the data obtained when an absorption spectrum was performed on purified apo-Met synthetase and holo-Met synthetase. Apo-Met synthetase contained insufficient Cbl to observe any significant peaks in the visible wavelength range. When the apo-Met synthetase was converted to holo-Met synthetase, the chelate in the running buffer. Met synthetase purified in the absence of oxygen was performed on purified apo-Met synthetase and holo-Met synthetase by Cob(I1)alamin.
synthetase by incubation with Cob(II)alamin followed by removal of unbound Cob(II)alamin by dialysis, major peaks at 311, 473, and 550 nm were observed. The spectrum for holo-Met synthetase did not precisely correlate with any of the spectra of coenzyme Cbls (14) but most closely resembled Cob(II)alamin (14) which has major peaks at 311 and 473 nm. Based on a molar extinction coefficient for Cob(II)alamin at 473 nm of 9400 (14), approximately 1.9 nmol of Cbl was bound to 250 μg (based on the amino acid composition, see below) of Met synthetase. Assuming a M, for Met synthetase of 160,000, this suggested that 1.9 nmol of Cbl were bound per 1.6 nmol of enzyme resulting in a stoichiometry of 1.2 mol of Cbl/mol of Met synthetase. These values were compatible with those obtained by direct assay of Cbl bound to holo-Met synthetase as well as binding of [35Co]Cob(II)alamin to Met synthetase.

Amino Acid Composition—The amino acid composition of Met synthetase is shown in Table III. After correction for recovery of the internal standard, the quantity of protein obtained by amino acid composition was 86% of that estimated by Lowry protein assays utilizing BSA as a standard. Analysis for carbohydrate was negative based upon a completely negative periodic acid-Schiff-stained gel with Met synthetase and a positive control utilizing transferrin which contains 6% carbohydrate.

Metal Analysis—We examined purified human Met synthetase for metal content by x-ray fluorescence as was performed with human methylnalonyl-CoA mutase (15). By this technique, we determined that 2 mol of iron/mol of Met synthetase was present. Although based on the quantity of holo-Met synthetase we expected to find 0.10 mol of cobalt (from the Cbl)/mol of purified Met synthetase, the cobalt could not be accurately determined because of interference by iron. By atomic absorption spectroscopy, we demonstrated the presence of 0.11 mol of cobalt/mol and 2 mol of iron/mol of purified Met synthetase and 1.1 mol of cobalt/mol of purified Met synthetase that was completely converted to holo-Met synthetase (Table V).

The Turnover of Crude and Purified Placental Met Synthetase—Since our preparation of placental Met synthetase had a low turnover number compared to the reported (3) turnover number of porcine kidney, we compared our assay and two other assays for activity of crude and purified Met synthetase from human placenta, porcine liver, and kidney (3, 22). It should be noted that neither of the latter methods of assay utilized Cbl in the assay system, while our assay (method 2, Table IV) measured activity both in the presence and absence of Cbl. The major difference between method 1 (porcine liver) and method 3 (porcine kidney) was that the length of incubation for method 1 was 60 min compared to 15 min for method 3 (3, 22).

Table IV contains the comparison of the different methods of assay in different tissues. Our method of assay (method 2) gave similar results to method 1 (reported for porcine liver) and method 3 (reported for porcine kidney). These specific activities, however, were strikingly lower than the specific activity reported (3) for porcine kidney.

Since turnover number has also been related to bound Cbl as determined with a microbiologic assay (22) and the value

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Amino acid analysis of methionine synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>Total residues exclusive of tryptophan and half-cystine</td>
</tr>
<tr>
<td>Aspartate</td>
<td>9.9</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.3</td>
</tr>
<tr>
<td>Serine</td>
<td>5.9</td>
</tr>
<tr>
<td>Proline</td>
<td>5.4</td>
</tr>
<tr>
<td>Glutamate</td>
<td>12.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.5</td>
</tr>
<tr>
<td>Valine</td>
<td>6.5</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>ND*</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.8</td>
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<tr>
<td>Leucine</td>
<td>9.6</td>
</tr>
<tr>
<td>Tyrosine</td>
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</tr>
<tr>
<td>Phenylalnine</td>
<td>4.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
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</tbody>
</table>

*ND, not determined.

<table>
<thead>
<tr>
<th>TABLE IV</th>
<th>Comparison of specific activity of methionine synthetase in various species and tissues by different methods of assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>Enzyme activity</td>
</tr>
<tr>
<td>Porcine liver, crude supernatant</td>
<td>0.045</td>
</tr>
<tr>
<td>Porcine kidney, crude supernatant</td>
<td>0.054</td>
</tr>
<tr>
<td>Human placenta, crude supernatant</td>
<td>0.063</td>
</tr>
<tr>
<td>Porcine kidney, 1877-fold purified</td>
<td></td>
</tr>
<tr>
<td>Porcine liver, 250-fold purified</td>
<td>(16.5)*</td>
</tr>
<tr>
<td>Porcine placenta, 1000-fold purified</td>
<td>1.33</td>
</tr>
</tbody>
</table>

* Assay performed as described in Ref. 22.
* Assay performed as described for purification of human placental methionine synthetase.
* Assay performed as described in Ref. 3.
* Indicates value reported for porcine liver (Ref. 22) and porcine kidney (Ref. 3).

<table>
<thead>
<tr>
<th>TABLE V</th>
<th>Determination of cobalamin by different assay methods on purified human placental methionine synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of assay</td>
<td>Cobalamin release</td>
</tr>
<tr>
<td>Radioisotope dilution (IF)*</td>
<td>Boiling, acid pH, KCN</td>
</tr>
<tr>
<td>Radioisotope dilution (R)*</td>
<td>Boiling, acid pH, KCN</td>
</tr>
<tr>
<td>Radioisotope dilution (R)</td>
<td>Pepsin, trypsin, pH, KCN</td>
</tr>
<tr>
<td>Cobalt by atomic absorption*</td>
<td></td>
</tr>
<tr>
<td>Bound [35Co]-Cob(II)alamin</td>
<td></td>
</tr>
</tbody>
</table>

* Refers to purified methionine synthetase prior to conversion to holo-enzyme by incubation with Cob(II)alamin. Approximately 90% of the purified methionine synthetase was in the apo form.
* IF refers to the use of intrinsic factor and R refers to the use of R-type cobalamin-binding protein in these assays.
* The assumption was made that each mol of cobalamin contained 1 mol of cobalt.
obtained was greater than 700, we analyzed the ability of a variety of Cbl assays to quantitate Cbl bound to purified Met synthetase. This data is shown in Table V. Although we did not employ the microbiologic assay, we compared the reported value for Cbl in crude porcine liver homogenate using the microbiologic assay (22) to the quantity of Cbl that we measured using a radioisotope dilution assay. The reported amount of Cbl/mg of protein in porcine liver homogenate was 0.51 pmol, and we detected 0.63 pmol of Cbl/mg with the radioisotope dilution assay. These results indicated that the radioisotope dilution assay was comparable to the microbiologic assay for detection of Cbl. Table V shows that the radioisotope dilution assay actually detected less than 5% of the Cbl bound to purified Met synthetase compared to the atomic absorption method or to [57Co]Cbl binding. Previous assays of Cbl bound to methylmalonyl-CoA mutase (15) indicated that this assay only detected a small percentage of the total Cbl present. The radioisotope dilution assay failed to accurately measure the Cbl bound to either of these enzymes despite the fact that both enzymes were subjected to boiling at pH 4.5 for 45 min in the presence of 0.8 mM potassium cyanide (10). Also shown in Table V is the fact that following treatment of Met synthetase with pepsin and trypsin digestion, the quantity of Cbl measured by the radioisotope dilution assay increased by 2-3-fold but was still considerably less than that detected by atomic absorption or [57Co]Cbl binding.

**DISCUSSION**

Mammalian Met synthetase has been purified from porcine kidney (3), bovine brain (27), and porcine liver (22). For the sake of comparison, the values listed below refer to enzyme units as nmol/min and specific activities as nmol/min/mg of protein. The specific activity of 1800-fold purified porcine kidney Met synthetase was 900 (3), 370-fold purified bovine brain Met synthetase was 187 (27), while 250-fold purified porcine liver had a specific activity of 16.5 (22) compared to the specific activity of our 935-fold purified human placental Met synthetase of 14. A major difference in specific activity also occurred in crude preparations among these tissues. While crude porcine kidney and crude bovine brain had specific activities of 0.5 (3, 27), crude porcine liver (22) and human placenta had specific activities of 0.07 and 0.02, respectively. The differences between crude porcine liver and porcine kidney were puzzling since the assays used for these two tissues were virtually identical. Assuming that mammalian Met synthetase from different sources have similar M, values, the turnover number, at least in crude tissues, appears to vary a great deal. Wide variation in specific activity with different assay methods and especially with variations in the reducing systems employed has been referred to in the past (25), and this appears to account for significant variations since crude bovine brain had a specific activity of 0.5 in the above studies but a specific activity of 0.05 (26) when assayed with a different assay system. Thus it seems that the specific activity of Met synthetase can vary as much as 10-fold in the same tissue. Our studies of crude human placentas revealed a 7-fold variation from placenta to placenta, indicating that factors other than the method of assay are also involved. Met synthetase protein levels appear to be regulated (25, 28) and responsive to dietary methionine (28) as well as other factors such as Cbl (5-7, 24). Assays of Met synthetase in a variety of human tissues have been reported, and the specific activities ranged from 0.009 (liver) (29, 30) to 0.076 (kidney) (29, 30) and from 0.06 (fibroblasts) (24) to 0.5 (fibroblast) (23) and 0.04 (lymphocytes) (20). To study the effect of the method of assay, we applied two types of anaerobic assays to crude and purified human placental Met synthetase as well as crude porcine liver and porcine kidney homogenates. Although the crude homogenates of liver and kidney were prepared as described (3, 22), we obtained markedly less specific activity than that reported (3) for crude porcine kidney, regardless of the method of assay, while our result with porcine liver was very similar to the value reported (22). Although we did not have purified porcine liver or kidney Met synthetase to assay, all three assays gave similar turnover numbers with purified human Met synthetase.

We also examined other possibilities that could explain our relatively low turnover number. Although purified porcine liver Met synthetase had a specific activity of 16.5, the turnover per bound Cbl was greater than 700. This value was significantly higher than would be predicted from purified porcine kidney Met synthetase with a specific activity of 900. Assuming a stoichiometry of Cbl binding of 1 mol of Cbl/mol of enzyme and a molecular weight of 150,000 for the porcine enzyme, the turnover per bound Cbl would be approximately 150. Earlier studies by us (15) indicated that Cbl bound to homogeneous human placental met synthetase was greatly underestimated by the usual assays employed to measure Cbl. While we did not employ the cumbersome microbiologic assay used to measure Cbl bound to porcine liver Met synthetase, our radioisotope dilution assay gave comparable results to those reported for crude porcine liver homogenate assayed with the microbiologic assay but greatly underestimated the Cbl bound to purified placental Met synthetase compared to the values obtained by atomic absorption or [57Co]Cbl binding (Table V). Thus it seemed likely that the high turnover of porcine liver Met synthetase compared to bound Cbl may have resulted from underestimation of the Cbl present. Indeed, the turnover of purified placental Met synthetase would have been as high as 120 had we used an assay comparable to the microbiologic assay to measure bound Cbl.

We also could not account for the low turnover by loss of enzyme activity. Since we recovered 20% of the enzyme activity, the turnover could be reduced by a maximum of 5-fold from the crude placental homogenate. Furthermore, a mixture of crude and purified Met synthetase produced results (data not shown) that were just additive suggesting that the activity of the purified met synthetase was not stimulated by a protein factor missing in purified enzyme but present in the crude homogenate. Although we could not rule out proteolysis in intact placenta or that a variety of uninhibited proteases were active, inclusion of a nonspecific protease inhibitor during the purification did not improve the turnover number of the purified enzyme.

It was also possible that we purified a Cbl-binding protein with a stoichiometry of Cbl binding of 1 mol/mol of protein that had a trace of Met synthetase activity. However, the purified Met synthetase differed from any of the known Cbl-binding proteins or the Cbl-dependent methylmalonyl-CoA mutase that have been purified and characterized in our laboratory (15, 31). Thus, the stoichiometry of Cbl binding and amino acid composition of purified Met synthetase was different from that of purified human placental methylmalonyl-CoA mutase (15), the M, and ability to bind CN-Cbl was different than purified transcobalamin II (31) or intrinsic factor (31), and the fact that purified R-type protein (haptocorrin) was a glycoprotein and could bind CN-Cbl (31) made contamination by this protein also seem unlikely.

Furthermore, the observations that apo-Met synthetase was functionally activated in direct proportion to binding of Cbl, the proportional loss of protein and activity from the active
peak of the high performance liquid chromatography column, and the correspondence of protein, Cbl, and enzyme activity in fractions of the gel filtration column strongly suggested that the same protein was providing the enzyme activity and Cbl binding. In summary, the above results suggest that human placental Met synthetase has a low turnover number in the range that has been described for a variety of other purified mammalian enzymes (32–35, 37). If human placental Met synthetase has a low turnover number, one might expect to find the relatively large quantities of enzyme protein that were, in fact, found in placental tissue. Compared to the quantity of methylmalonyl-CoA mutase present in human placenta (15), we would estimate that the majority of cobalammin binding in cells would be to Met synthetase. When human fibroblasts have been incubated with \(^{57}\)Co-Cbl followed by studies of intracellular distribution of bound Cbl between Met synthetase and mutase, a striking predominance of Cbl bound to Met synthetase was observed (23, 24, 38). This observation was compatible with the quantities of Met synthetase enzyme protein compared to the quantities of methylmalonyl-CoA mutase enzyme protein (15) present in human placenta. The data from fibroblasts as well as the apparent low turnover number of Met synthetase from other crude human tissues also suggests that high levels of Met synthetase enzyme protein with a low turnover number is not a situation unique to human placenta compared to other human tissues. However, it is also possible that human Met synthetase has a higher turnover number in vivo and that the turnover number was lowered by removing the enzyme from its natural environment. If human Met synthetase has a low turnover number in vivo compared to other mammalian Met synthetase, this may, in some way, be a reflection of the uniqueness of megakaryoblastic anemia in humans compared to other animals.

The results of a number of other studies of the purified Met synthetase were also of interest. The absorption spectrum, while not identical to that described for any form of Cbl, most closely resembled that described for Cob(II)alamin. It was also of interest that the apo-Met synthetase appeared not to bind CN-Cbl but stoichiometrically bound Cob(II)alamin. This observation was compatible with the suggestion that apo-Met synthetase binds Cob(II)alamin in preference to other Cbl forms in human fibroblasts (23, 24). We are presently studying the binding of other forms of Cbl including methylcobalamin and 5'-deoxyadenosylcobalamin to apo-Met synthetase.

Protein-staining bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a major M, band of 160,000 was present on the unreduced gel, but traces of M, 80,000 and 70,000 bands also appeared to be present. In contrast, when the protein was reduced by boiling in 2-mercaptoethanol, these bands disappeared and bands of M, 90,000, 45,000, and 35,000 appeared, suggesting that the subunit structure of human Met synthetase was complex. Similar complexity as well as uncertainty about the subunit structure of highly purified bacterial Met synthetase also exists (25). Furthermore, we could not be certain that the smaller M, bands represented subunits of Met synthetase since the M, 35,000 band appeared to be present in greater quantities based on intensity of staining. It was possible, for example, that the apparent subunit structure revealed on the reduced SDS gel occurred as a result of partial proteolysis. Finally, although our studies are preliminary, it appeared that the purified human Met synthetase contained 2 mol of iron/mol of enzyme protein. This result was also interesting compared to bacterial Met synthetase (21) where enzyme R and P components were described which were suggested (21) to function in the reduction of Cbl bound to Met synthetase (the M component). The R component had an M, of approximately 29,000 and the F component a M, of approximately 30,000 while the M component that contained bound Cbl had an M, of 90,000. Since purified human Met synthetase appeared to contain bound iron, it is possible that the lower M, subunits bind iron and could participate in similar oxidation-reduction reactions for the human enzyme.

In summary, human Met synthetase is a complex enzyme that catalyzes several reactions. The availability of the purified enzyme will provide a means of determining: the nature of interaction of a variety of forms of Cbl with the enzyme; the mechanism of reduction of Cbl; the mechanism of methyl transfer; and the role of Met synthetase in folate uptake and intracellular folate metabolism.

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REFERENCES

Purification of Human Methionine Synthetase


