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Biochemistry

Glycine *N*-methyltransferase is a folate binding protein of rat liver cytosol

(*S*-adenosylmethionine/one-carbon metabolism/methylation)

ROBERT J. COOK* AND CONRAD WAGNER*†

*Department of Biochemistry, Vanderbilt University, Nashville, TN 37232; and †Veterans Administration Medical Center, Nashville, TN 37203

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ABSTRACT **A comparison of the amino acid compositions of one of the folate-binding proteins of rat liver cytosol, folate-binding protein–cytosol II, and that of glycine *N*-methyltransferase (*S*-adenosyl-L-methionine:glycine methyltransferase, EC 2.1.1.20) from the same source indicated a great deal of structural homology between the two proteins. Antiserum prepared against the purified folate-binding protein almost completely inactivated the enzyme activity in crude liver cytosol. Purification of glycine *N*-methyltransferase resulted in the separation of two enzyme species, one that contained bound folate and one that did not. Each species was homogeneous, as judged by NaDodSO$_4$/polyacrylamide gel electrophoresis, and they migrated identically.**

The enzyme glycine *N*-methyltransferase (*S*-adenosyl-L-methionine:glycine methyltransferase, EC 2.1.1.20) catalyzes the synthesis of sarcosine from *S*-adenosylmethionine and glycine. It was first described by Blumenstein and Williams (1) in guinea pig liver and was subsequently found in the livers of a large number of mammalian species (2). Heady and Kerr (3) purified the enzyme from rabbit liver and showed that it was a glycoprotein with a M_r of 133,500, as determined by equilibrium centrifugation. They found a minimum of three nonidentical subunits with M_r values between 27,000 and 33,000 by NaDodSO$_4$/polyacrylamide gel electrophoresis. More recently, the enzyme was purified from rat liver and shown to have properties significantly different from the rabbit liver enzyme (4). Rat liver glycine *N*-methyltransferase has a M_r of 132,000 by sedimentation equilibrium, consists of four identical subunits, each with a M_r of 31,500, and is not a glycoprotein.

We have noted that the properties of the rat liver glycine *N*-methyltransferase as described by Ogawa and Fujioka (4) closely resemble those of a folate-binding protein that has been purified in our laboratory (5–7). This protein was identified in rat liver cytosol as the second folate-binding protein to elute from a Sephadex G-150 column and was named folate-binding protein–cytosol II (FBP–CII). The purified protein contained tightly bound 5-methyltetrahydropteroylpentaglutamate (5-CH$_3$-H$_4$PteGlu$_5$) and has an estimated M_r of 150,000 by both gel chromatography and by sucrose-density centrifugation. NaDodSO$_4$/polyacrylamide gel electrophoresis showed a single band with a M_r of ≈32,000. The function of FBP–CII has not been determined, but it was shown to be neither methylenetetrahydrofolate reductase [5-methyltetrahydrofolate:(acceptor) oxidoreductase, EC 1.1.99.15] nor methionine synthase (tetrahydropteroylglutamate methyltransferase, 5-methyltetrahydropteroyl-L-glutamate:L-homocysteine *S*-methyltransferase, EC 2.1.1.13), the two enzymes responsible for the synthesis and utilization, respectively, of 5-CH$_3$-H$_4$PteGlu$_5$.

In this report, we provide evidence to show that FBP–CII and glycine *N*-methyltransferase from rat liver are the same or very similar proteins.

EXPERIMENTAL PROCEDURES

Materials. Generally labeled folic acid ([G-^{3}H]PteGlu) was obtained from Moravek Biochemicals (City of Industry, CA). Radioactive *S*-adenosyl-L-[*methyl*-^{3}H]methionine was obtained from Amersham and unlabeled *S*-adenosyl-L-methionine was obtained from Sigma. DEAE-cellulose (DE-52) and carboxymethylcellulose (CM-cellulose) were purchased from Whatman. Sephadex G-150 was obtained from Pharmacia and hydroxylapatite was obtained from Clarkson Chemical (Williamsport, PA) as Hypatite C. The marker proteins used for the polyacrylamide gel electrophoresis were obtained from Bio-Rad. Acid-washed charcoal was prepared from Darco G-60 as described (8). All other chemicals were the highest grade commercially available.

Animals. Male Sprague–Dawley rats (200–350 g) were obtained from Harlan Laboratories. They were fed *ad lib* Wayne Lab Blox. Rats were injected 3 times intraperitoneally at 72, 48, and 24 hr with 10 μCi (1 Ci = 37 GBq) of [G-^{3}H]PteGlu before they were killed.

Antiserum. Antiserum to FBP–CII was prepared as described (7).

Measurement of Glycine *N*-Methyltransferase Activity. A rapid assay using acid-washed charcoal to adsorb unreacted radioactive *S*-adenosylmethionine was developed. The reaction was carried out in 1.5 ml of capped polyethylene centrifuge tubes (Brinkmann). The reaction mixture contained 20 μl of 0.5 M Tris·HCl, pH 7.4/20 μl of 10 mM glycine/20 μl of 1 mM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1.0 μCi/μmol), and sample in a final volume of 0.1 ml. Incubation was carried out at 25°C for 15 min. The reaction was stopped by the addition of 50 μl of cold 10% trichloroacetic acid followed by 250 μl of a charcoal suspension (38 mg/ml in 0.1 M acetic acid). The tubes were then incubated at 0°C for 15 min and centrifuged for 2 min in a Brinkmann tabletop microfuge. Two hundred microliters of the supernatant was added to 5 ml of aqueous counting scintillant (Amersham) and assayed. All assays were carried out in duplicate. Blank values obtained in the absence of glycine were subtracted to give net counts. Enzyme activity was expressed as nmol of sarcosine formed in 15 min and was proportional to the amount of enzyme, provided the rate of synthesis did not exceed 1.8 nmol of sarcosine in 15 min. The product was identified as sarcosine by high-pressure liquid chromatography on an Aminex A-9 cation exchange column (Bio-Rad). Elution was carried out isocratically with 0.325 M sodium citrate buffer (pH 3.28). When this method was run in parallel with that used by Ogawa and Fujioka (4), approximately the same values were obtained.

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Abbreviations: FBP–CII, folate binding protein-cytosol II; 5-CH$_3$-H$_4$PteGlu, 5-methyltetrahydropteroylglutamic acid (5-methyltetrahydrofolate); PteGlu, pteroylglutamic acid (folic acid); 5-CH$_3$-H$_4$PteGlu$_5$, 5-methyltetrahydropteroylpentaglutamic acid.

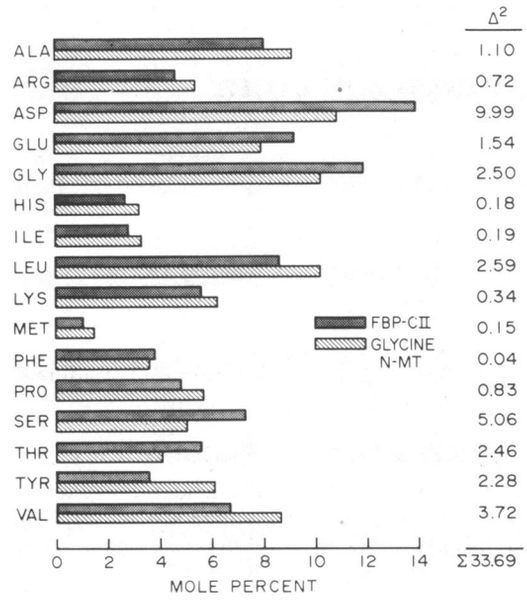

FIG. 1. Relationship between the amino acid compositions of FBP–CII and glycine *N*-methyltransferase (Glycine N-MT). The methods for calculating Δ^2 and Σ (SΔQ) are described in the text.

Other Methods. Protein was measured by a modification of the Bradford method as described (9). Polyacrylamide gel electrophoresis was carried out under denaturing conditions in the presence of $NaDodSO_4$ according to the method of Laemmli (10), using a 3% stacking gel at pH 6.8 and a 9% separating gel at pH 9.8. Liquid scintillation counting was carried out by dissolving samples in aqueous counting scintillant (Amersham) and assaying them in a Searle Isocap or Tracor counter. Corrections for quenching and efficiency were made by the external standard ratio method.

RESULTS

Amino Acid Composition of FBP–CII and Glycine *N*-Methyltransferase. The amino acid compositions of FBP–CII as published by Suzuki and Wagner (6) and that of glycine *N*-methyltransferase as published by Ogawa and Fujioka (4) were compared using the method of Marchalonis and Weltman (11). This method compares the relative abundance of the 16 most commonly measured amino acids in two proteins as an index of relatedness. Each amino acid is expressed as the mol percent of the total number of amino acids in each protein. The difference in mol percent between the two proteins for each amino acid is squared, and these values are then summed to provide the parameter SΔQ. Marchalonis and Weltman compared over 100 proteins by this method. In 98% of the comparisons, unrelated proteins differed by >100 SΔQ units. In no case did proteins thought to be unrelated differ by <50 SΔQ units. Fig. 1 shows the relative abundance of amino acids in FBP–CII and glycine *N*-methyltransferase in graphic form. The value of 33.70 for SΔQ (Σ) suggests a high degree of relatedness for the two proteins.

Table 1. Inhibition of glycine *N*-methyltransferase activity by immune antiserum

Serum	Enzyme activity, nmol/min per mg of protein
Preimmune	1.44
Immune	0.04

Unfractionated rat liver cytosol (20 μl containing 58 μg of protein) was incubated with 30 μl of undiluted serum for 3 hr at 37°C. The serum was either immune antiserum raised against purified folate-binding protein FBP–CII or the preimmune serum from the same rabbit. Reaction mixtures were then assayed for glycine *N*-methyltransferase activity.

Immunologic Similarity Between Folate-Binding Protein, FBP–CII, and Glycine *N*-Methyltransferase. Antiserum prepared against purified FBP–CII inhibited the glycine *N*-methyltransferase activity in crude rat liver cytosol. The antiserum had been prepared previously for use in a radioim-

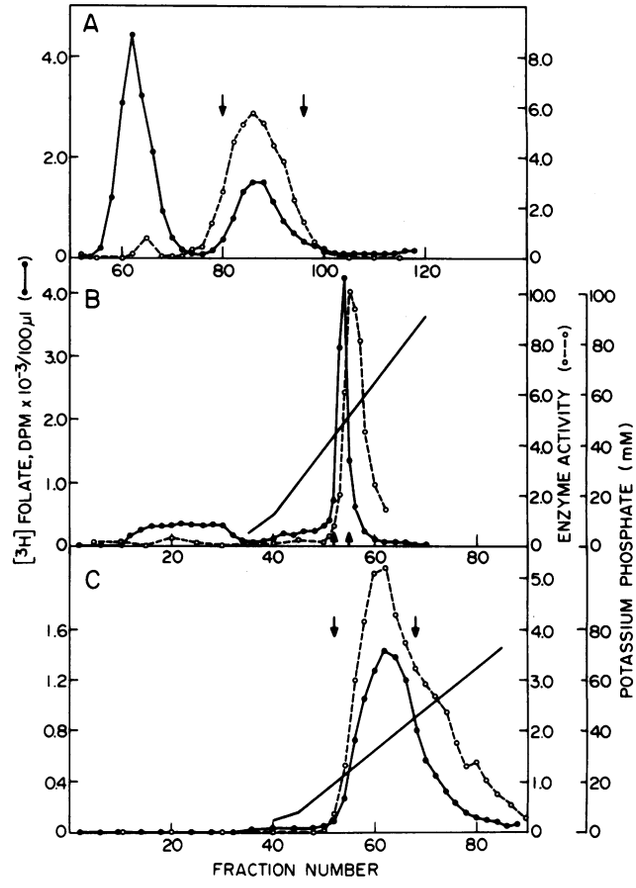

FIG. 2. Purification of glycine *N*-methyltransferase. (*A*) Sephadex G-150 chromatography. Cytosol (40 ml) was applied to a Sephadex G-150 column (87 × 5 cm) equilibrated with 10 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol/1 mM sodium azide. The column was eluted at 1 ml/min with equilibration buffer, and 10-ml fractions were collected. The fractions between the arrows were pooled, adjusted to pH 6.0, concentrated, and dialyzed against 5 mM potassium phosphate, pH 6.0/10 mM 2-mercaptoethanol/1 mM sodium azide. (*B*) Carboxymethylcellulose chromatography. The concentrated dialyzed fractions from the Sephadex G-150 column were applied to a carboxymethylcellulose column (30 × 3 cm) equilibrated with dialysis buffer. The column was washed with 100 ml of dialysis buffer and then eluted, at 0.65 ml/min, with a linear gradient of 5–300 mM potassium phosphate, pH 6.0/10 mM 2-mercaptoethanol/1 mM sodium azide (total vol, 500 ml). Fractions of 8 ml were collected. The fractions between the arrows were pooled, adjusted to pH 7.0, concentrated, and dialyzed against 5 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol/1 mM sodium azide. (*C*) Hydroxylapatite chromatography. The concentrated dialyzed peak from the carboxymethylcellulose column was applied to a hydroxylapatite column (12 × 2.1 cm) equilibrated with 5 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol/1 mM sodium azide. The column was washed with 20 ml of equilibration buffer and then eluted at 0.5 ml/min with a linear gradient of 5–100 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol/1 mM sodium azide (total vol, 200 ml). Fractions of 3 ml were collected. The fractions between the arrows were pooled and used for further studies.

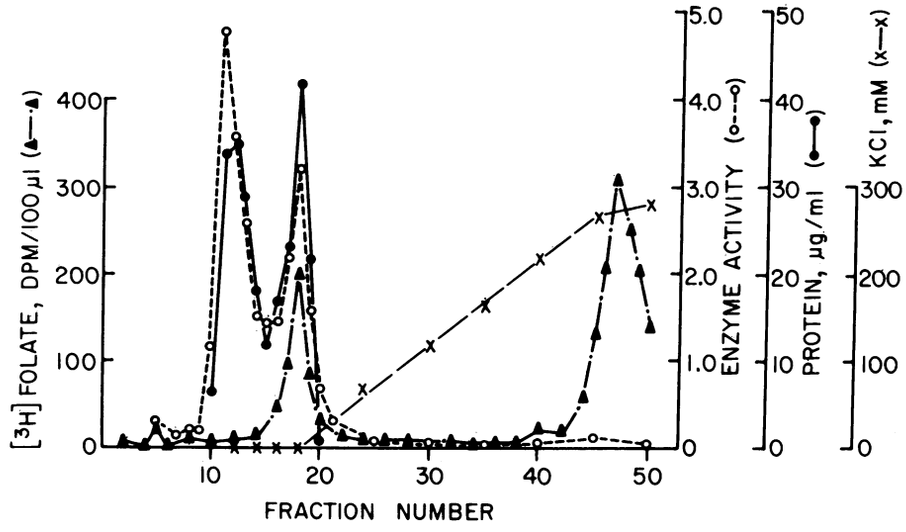

FIG. 3. DEAE-cellulose column chromatography of glycine *N*-methyltransferase. Purified enzyme was concentrated to a final vol of 15.2 ml in an Amicon filtration apparatus with a PM30 filter. The concentrate was applied to a 5 × 1.5 cm column of DEAE-cellulose equilibrated with 10 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol/1 mM sodium azide. The column was washed with 40 ml of equilibration buffer and then a linear gradient of KCl (0–0.5 M) in equilibration buffer was applied (total vol, 200 ml). The column was eluted at 0.5 ml/min, and 3-ml fractions were collected.

munoassay to measure FBP–CII in various tissues (7). Table 1 shows that 97% of the enzyme activity was inhibited by the specific antiserum when compared with serum from the same rabbit prior to immunization.

Purification of Folate-Binding Protein, FBP–CII, with Glycine *N*-Methyltransferase Activity. The procedure used for purification of FBP–CII uses intraperitoneal injection of [^{3}H]PteGlu 72, 48, and 24 hr prior to isolation in order to label the binding proteins *in vivo*. In this way, FBP–CII contains tightly bound [^{3}H]5-CH_3-H_4PteGlu$_5$, and purification is followed by monitoring the radioactivity during the various steps. These purification steps were designed to preserve the binding between the radioactive ligand and the protein (6, 7). The enzyme activity and radioactive ligand cochromatograph on a Sephadex G-150 column (Fig. 2*A*). Carboxymethylcellulose chromatography at pH 6.0 (Fig. 2*B*) shows that the radioactive ligand elutes on the leading edge of the enzyme activity peak. Chromatography on hydroxylapatite at pH 7.0 (Fig. 2*C*) of the protein containing the bound radioactive ligand shows cochromatography of the enzyme activity and the radioactive ligand. This preparation was then chromatographed sequentially on the following columns: carboxymethylcellulose, pH 7.0; hydroxylapatite, pH 7.8; hydroxylapatite, pH 6.0; and carboxymethylcellulose, pH 6.0. On all of these columns only a single peak was seen in which the bound radioactive ligand, protein, and glycine *N*-methyltransferase activity cochromatographed exactly. This enzyme preparation was then chromatographed on a DEAE-cellulose column (Fig. 3), which resulted in the enzyme activity being split into two peaks. The first peak had no bound folate, while the second peak of activity cochromatographed with the radioactive folate. In addition, a large radioactive peak eluted from the column with a higher salt concentration. This latter peak had no protein associated with it and presumably was a radioactive folate that had been dissociated on the column. All of the protein was associated with the two peaks of enzyme activity. In another experiment (not shown), both peaks of enzyme activity were able to rebind radioactive 5-CH_3-H_4PteGlu$_5$ *in vitro*. Polyacrylamide gel electrophoresis of samples from each peak of enzyme activity showed a single band with the same mobility (Fig. 4). These were estimated to have a M_r of 35,000.

DISCUSSION

The evidence presented here strongly suggests that the folate-binding protein of rat liver cytosol, FBP–CII, and glycine *N*-methyltransferase are one and the same protein. Two species of enzyme were separated, however, by DEAE-cellulose chromatography; one that retained tightly bound radioactive folate and one that did not (Fig. 3). It is unclear whether these two species represent different forms of the protein and have different affinities for the folate ligand, or

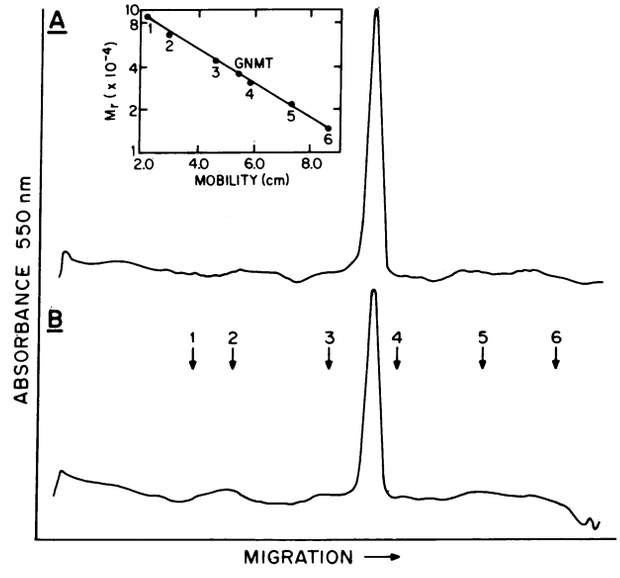

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of glycine *N*-methyltransferase (GNMT). Protein was treated with 1% NaDodSO$_4$ and 1% 2-mercaptoethanol, heated in a boiling water bath for 5 min, and electrophoresed in a 0.15 × 10 × 14 cm slab gel. After staining with Coomassie blue, the gel was destained and scanned at 550 nm with a Corning model 740 densitometer. The arrows indicate the positions to which the following marker proteins migrated: 1, phosphorylase B; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, soybean trypsin inhibitor; 6, lysozyme. (*A*) Tube 11 from DEAE-cellulose (Fig. 2; 3.5 µg). (*B*) Tube 18 from DEAE-cellulose (Fig. 2; 4.2 µg).

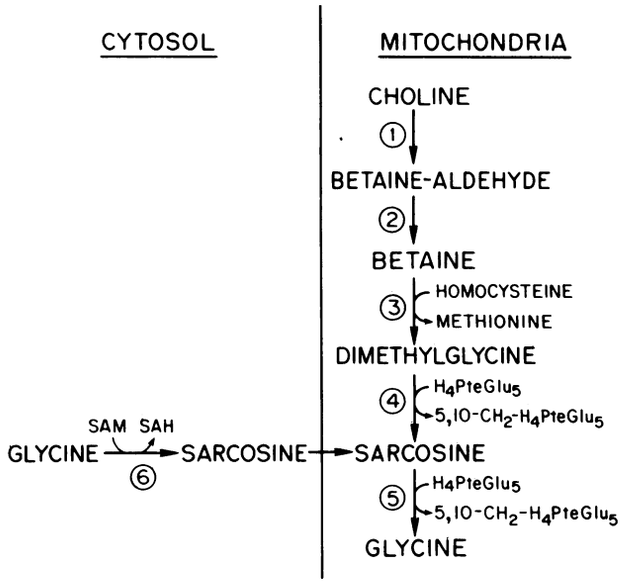

FIG. 5. Pathway of sarcosine formation. The steps are carried out by the indicated enzymes: 1, choline dehydrogenase (EC 1.1.99.1); 2, betaine-aldehyde dehydrogenase (EC 1.2.1.8); 3, betaine-homocysteine methyltransferase (EC 2.1.1.5); 4, dimethylglycine dehydrogenase (EC 1.5.99.2); 5, sarcosine dehydrogenase (EC 1.5.99.1); 6, glycine *N*-methyltransferase (EC 2.1.1.20).

whether partial dissociation of the bound folate had taken place before application to the column, which then resolved the species that contained the bound folate from the one that did not. With regard to the latter possibility, it is likely that the folate ligand is not all 5-CH_3-$H_4PteGlu_5$. Although our previous studies indicated that the majority of folate bound to FBP–CII was the pentaglutamate, a small amount of another species also was bound (6). Its properties suggested that it might have a shorter polyglutamate chain. If shorter chain length 5-CH_3-$H_4PteGlu$ polyglutamates are bound to the protein, they might be dissociated more easily, thus giving rise to protein species that have retained or lost the folate ligands. The fact that the species of enzyme with bound folate polyglutamate eluted from the carboxymethylcellulose column at pH 6.0 (Fig. 2*B*) before the main portion of the glycine *N*-methyltransferase activity, and eluted from the DEAE-cellulose column (Fig. 3) after the enzyme species without bound folate polyglutamate ligand, is consistent with the expected chromatographic behavior of the protein with the additional negative charges of the folate polyglutamate ligand. Since virtually all the enzyme activity in the crude preparation of the cytosol was inactivated by the antiserum, it seems clear that all the species of glycine *N*-methyltransferase are closely related to FBP–CII.

If bound 5-CH_3-$H_4PteGlu_5$ is not necessary for the activity of glycine *N*-methyltransferase activity, why is most of the 5-CH_3-$H_4PteGlu$ polyglutamate in the cell bound to this enzyme (12), and what function does it perform there? Glycine *N*-methyltransferase is found in abundant quantities in liver. It has been estimated that this enzyme may account for as much as 3% of the soluble protein of rabbit liver (3), and FBP–CII was shown to make up 0.5% of the soluble protein in rat liver cytosol (7). It is also present in guinea pig liver. Although glycine *N*-methyltransferase has not been measured directly in human liver, it is assumed to be present, and the disposition of excess methionine in humans is believed to involve the conversion to *S*-adenosylmethionine followed by methylation of glycine to sarcosine (13). The high activity of this enzyme was shown to be responsible for the failure to detect tRNA methyltransferase activity in crude tissue extracts (2). The relative amounts of glycine *N*-methyltransferase and tRNA methyltransferase are 3520:1 in rat liver cytosol (2), and the tissue levels of glycine and *S*-adenosylmethionine are such that formation of sarcosine is favored in crude extracts. In spite of the abundant activity of glycine *N*-methyltransferase, no clear role for the enzyme has emerged, and it has been suggested to play a role in modulating the relative levels of *S*-adenosylmethionine and *S*-adenosylhomocysteine in the cell (2, 4).

The synthesis of sarcosine can also arise in liver from choline breakdown. This occurs exclusively in liver mitochondria by a pathway shown in Fig. 5. The oxidation of dimethylglycine to sarcosine is carried out by dimethylglycine dehydrogenase [*N*,*N*-dimethylglycine:(acceptor) oxidoreductase (demethylating), EC 1.5.99.2], and the oxidation of sarcosine to glycine is carried out by sarcosine dehydrogenase [sarcosine:(acceptor) oxidoreductase (demethylating), EC 1.5.99.1]. It is of interest to note that both of these enzymes contained tightly bound folate and were originally identified as the folate-binding proteins of mitochondria (14–16). Thus, any possible regulation of glycine *N*-methyltransferase by the 5-CH_3-$H_4PteGlu_5$ ligand may relate to the oxidation of one-carbon units.

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