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Identification of folate binding protein of mitochondria as dimethylglycine dehydrogenase

(flavoprotein/sarcosine dehydrogenase/tetrahydrofolate)

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ABSTRACT **The folate-binding protein of rat liver mitochondria [Zamierowski, M. & Wagner, C. (1977) *J. Biol. Chem.* 252, 933–938] has been purified to homogeneity by a combination of gel filtration, DEAE-cellulose, and affinity chromatography. This protein was assayed by its ability to bind tetrahydro[^{3}H]folic acid *in vitro*. The purified protein contains tightly bound flavin and has a molecular weight of about 90,000 as determined by sodium dodecyl sulfate electrophoresis. This protein also displays dimethylglycine dehydrogenase [*N*,*N*-dimethylglycine: (acceptor) oxidoreductase (demethylating), EC 1.5.99.2] activity which copurifies with the folate-binding activity. It is suggested that the role of the tetrahydrofolic acid is to accept the formaldehyde produced during the course of the reaction.**

Three years ago this laboratory reported the presence of a group of cellular folate-binding proteins in rat liver (1) on the basis of the incorporation of intraperitoneally administered [^{3}H]folic acid (PteGlu) into high molecular weight forms. By using chromatography on Sephadex G-150, three separate binding proteins were identified in the cytosol and a fourth was found in extracts of the rat liver mitochondria. Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) activity was associated with the protein from cytosol having an estimated molecular weight of 25,000. None of the other protein fractions could be shown to exhibit any of a number of enzyme activities known to participate in folic acid metabolism.

We have now succeeded in purifying the folate-binding protein from mitochondria to a stage of apparent homogeneity. This protein was previously shown (1) to have an apparent molecular weight of 90,000 by gel filtration of the crude mitochondrial extract. Further purification has demonstrated that it contains tightly bound flavin and has dimethylglycine dehydrogenase [*N*,*N*-dimethylglycine: (acceptor) oxidoreductase (demethylating), EC 1.5.99.2] activity.

MATERIALS AND METHODS

Materials. 2-Mercaptoethanol was obtained from Eastman Kodak, 2,6-dichlorophenolindophenol and dimethylglycine hydrochloride were from Sigma, phenazine methosulfate was from Mann Research Laboratories (Orangeburg, NY), sarcosine hydrochloride was from Calbiochem, DEAE-cellulose (DE-52) was from Whatman, and aminohexyl-Sepharose-4B was from Pharmacia. 5-Formyltetrahydrofolic acid was obtained as the calcium salt from Lederle Laboratories (Pearl River, NY) and pterin-6-carboxylic acid was from Aldrich. All other chemicals were the highest grade commercially available.

Preparation of Tetrahydro[^{3}H]folic Acid (H_4[^{3}H]PteGlu). [3′,5′,7,9-^{3}H]PteGlu, potassium salt (20 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was obtained from Amersham. Unlabeled PteGlu (Sigma) was added to adjust the specific activity to 20 μCi/μmol. H_4[3′,5′,7,9-^{3}H]PteGlu was synthesized by chemical reduction with $NaBH_4$ (2). To 0.70 ml of 0.066 M Tris·HCl at pH 7.8 was added 0.30 ml of a solution containing 0.40 μmol (8 μCi) of [^{3}H]PteGlu. This solution was stirred in the dark under nitrogen at room temperature, and 0.25 ml of $NaBH_4$ (120 mg/ml) was added. Disappearance of yellow color and a shift of the absorption maximum from 282 nm to 296 nm (3) indicated the formation of H_4PteGlu. After 45 min, the pH was adjusted to 7.5 with 5 M acetic acid, 14 μl of 2-mercaptoethanol was added, and the volume was adjusted to 2 ml with water. This solution was used, without purification, for binding assays and was stored under nitrogen at −20°C. Analysis of purity by ion exchange chromatography (0.8 × 8 cm column of DEAE-cellulose equilibrated with 0.1 M potassium phosphate, pH 6.1/0.1 M 2-mercaptoethanol and eluted with 0.3 M KCl in the equilibrating buffer) showed it to be 60–65% pure.

Separate experiments established that the likely contaminants in this preparation, PteGlu, *p*-aminobenzoylglutamic acid, and pterin-6-carboxylic acid, did not interfere with the binding assay at the possible levels present.

Centrifugal Desalting Procedure. Rapid desalting of small volumes of liquid by centrifugation through small columns of gel filtration medium (4) has been used to measure ligand binding to macromolecules (5). Bio-Gel P-10 (Bio-Rad) was equilibrated with buffer (10 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol). The gel was transferred to small disposable plastic columns (4.5 ml screening tubes, Whale Scientific, Denver, CO) to yield 2 ml of gravity-packed gel (3 × 0.9 cm). Columns were inserted into receiving tubes (16 × 100 mm glass test tubes) and were readied for sample application by centrifugation at 620 × *g* for 3 min in a Sorvall GLC-1 centrifuge. The volume of the packed gel decreased by about 50% during this centrifugation. The columns were transferred to clean receiving tubes and the sample to be desalted (about 0.2 ml) was applied to the gel surface. The centrifugation was repeated and the desalted sample was recovered from the receiving tube. The gel columns were regenerated by washing twice with 1.5-ml portions of buffer. Washing was facilitated by centrifugation at 35 × *g* for 3 min.

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Abbreviations: PteGlu, pteroylglutamic acid (folic acid); H_4PteGlu, 5,6,7,8-tetrahydropteroylglutamic acid (tetrahydrofolic acid).

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Folate-Binding Assay. This method utilizes the centrifugal desalting procedure described above. All steps except the centrifugation were carried out with the receiving tubes and columns chilled in an ice bath. A 10 μM solution of H_4[3′, 5′,7,9-^{3}H]PteGlu (20 μCi/μmol) was prepared immediately before use by diluting a 200 μM solution with 10 mM potassium phosphate, pH 7.0/10 mM 2-mercaptoethanol; 20 μl of this solution was placed in each receiving tube. Protein samples (0.2 ml) were applied to the gel columns and centrifuged, thus transferring the desalted protein into the labeled ligand. This initial desalting eliminated any possible effects of buffer composition on binding. Column fractions from affinity chromatography were desalted twice to eliminate inhibition of binding by the PteGlu used to elute the protein. After incubation of the desalted protein with the labeled ligand for 10–20 min (enough time to regenerate the gel columns) the incubation mixture was again put through the centrifugal desalting procedure to remove unbound ligand.

Radioactivity that was centrifuged through the column was indicative of H_4PteGlu binding. Binding appeared to take place very rapidly under the conditions of the assay. Identical binding was observed when the 10- to 20-min incubation period was omitted. In other experiments, H_4[^{3}H]PteGlu was bound to protein and stored for several days at 5°C in the presence of 10 mM 2-mercaptoethanol. After dissociation by heating in a boiling water bath in the presence of 1% sodium ascorbate, >90% of the labeled material chromatographed as H_4PteGlu. This suggests that the normally labile H_4PteGlu is protected from aerobic oxidation when it is bound to protein. Binding was proportional to the amount of sample added in the range 0–800 dpm of recovered bound radioactivity, or up to about 10% of the total label in the incubation mixture. Sample dilutions in the linear range were used when the various purification stages were assayed. Control experiments established that in the absence of protein, 0.5% (44 dpm) or less of the applied radioactive ligand appeared in the desalted fraction. Recovery of protein averaged 83% for one passage through the column and 63% for the entire binding assay.

Because this assay was used as a rapid method to obtain relative values for binding activity, activities are reported uncorrected for this loss and possible additional loss of binding during the desalting procedure. A stoichiometry of 1 mol of ligand per mol of protein for the purified material has been established by other methods (unpublished data).

Dimethylglycine/Sarcosine Dehydrogenase Assay. Dimethylglycine or sarcosine dehydrogenase (EC 1.5.99.1) activity was measured by the rate of 2,6-dichlorophenolindophenol reduction at 600 nm (molar absorption coefficient, 21,000). The method of Hoskins and Bjur (6) was used with minor modifications. Each cuvette contained 200 μmol of Tris buffer (pH 8.3), 0.06 μmol of 2,6-dichlorophenolindophenol, 50 μg of phenazine methosulfate, 5 μmol of KCN (pH 8.0), 5 μmol of dimethylglycine or sarcosine (pH 7.5), enzyme, and water to a volume of 1.0 ml. After an initial equilibration period at 25°C, the reaction was initiated by the addition of substrate. The rate of dye reduction 0.5–2.0 min after the addition of substrate was proportional to the amount of enzyme and was used as the measure of enzymatic activity. After 2 min, a marked decrease in activity was observed. Similar departures from linearity, dependent on pH and phenazine methosulfate concentration, have been observed by others (6).

Other Methods. Protein was measured by the method of Bradford (7) with bovine albumin (Sigma) as a standard; 3 ml of dye reagent was mixed with 0.1-ml samples and the absorbance at 595 nm was determined. NaDodSO$_4$/polyacrylamide gel electrophoresis was conducted according to Laemmli (8), using a 9% separating gel at pH 8.8 and a 3% stacking gel at pH 6.8. Gels were stained in 0.05% Coomassie blue in 14% methanol/8% acetic acid and destained by diffusion in 10% methanol/10% acetic acid.

RESULTS

Copurification of Folate-Binding Activity and Dimethylglycine Dehydrogenase Activity. We have previously shown (9) that H_4PteGlu and a single polyglutamate species of H_4PteGlu are the forms of folate found associated with the mitochondrial binding protein after passage through a Sephadex G-150 column. The polyglutamate species has been identified as the pentaglutamate, H_4PteGlu$_5$ (unpublished data). It was subsequently learned that H_4[^{3}H]PteGlu would bind rapidly to this protein *in vitro*; this formed the basis for the folate binding assay used here. By using this assay, a three-step procedure for purification of this protein has been developed: gel filtration chromatography on Sephadex G-150, DEAE-cellulose chromatography, and affinity chromatography. Details of the purification procedure will be published separately.

After gel filtration chromatography on Sephadex G-150, the material containing the binding activity was pooled, concentrated to about 1/10th volume, and passed over a DEAE-cellulose column. Most of the binding activity was adsorbed to the column. Elution with a gradient of 0–0.5 M KCl resulted in a major peak of binding activity being eluted at a conductivity of 7 mmho. A second minor peak of binding activity was eluted at 16 mmho. The major peak of binding activity was pooled, concentrated to about 1/10th volume, and equilibrated with 0.01 M phosphate buffer at pH 7.8. This was then applied to an affinity column containing 5-formyltetrahydrofolic acid linked to aminohexyl-Sepharose-4B through the carboxyl groups. Most of the folate binding activity was adsorbed to the

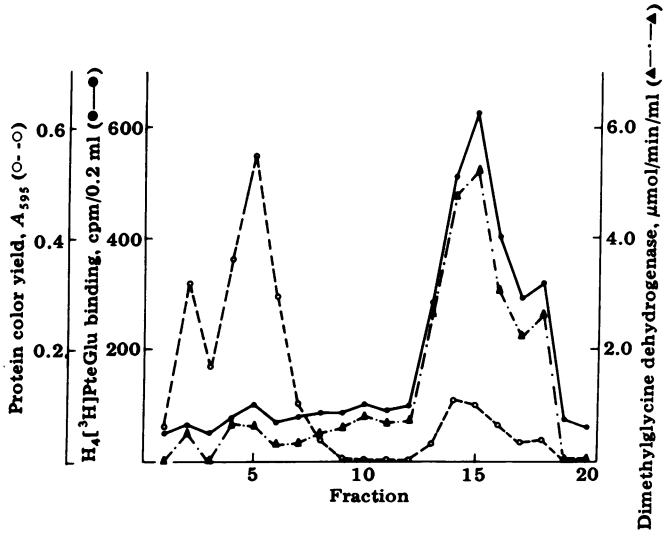

FIG. 1. Copurification of folate-binding activity and dimethylglycine dehydrogenase activity on a folate affinity column. 5-Formyltetrahydrofolic acid was linked to aminohexyl-Sepharose-4B by the water-soluble carbodiimide method (10). Analysis indicated that 37 μmol of 5-formyltetrahydrofolic acid was coupled to the Sepharose which had a maximum of 40 μmol of aminohexyl groups (manufacturer's specification). A 1 × 3 cm column of this material was equilibrated with 10 mM potassium phosphate (pH 7.8). After application of the sample, a KCl gradient (0–1 M) in the equilibrating buffer was applied (total volume, 100 ml). At fraction 12, a PteGlu gradient (0–20 mM in 1 M KCl and equilibrating buffer) was begun (total volume, 100 ml). The flow rate was 0.7 ml/min and the fraction size was 9.3 ml. During application of the PteGlu gradient, the flow was stopped for 15 min between fractions. The flow was interrupted for several hours prior to the collection of fraction 18.

Table 1. Purification of mitochondrial folate-binding protein

Step	Vol., ml	Total protein, mg	Dehydrogenase activity,* units/mg: Dimethylglycine	Dehydrogenase activity,* units/mg: Sarcosine	H_4PteGlu binding activity†: pmol/mg	H_4PteGlu binding activity†: Recovery, %	H_4PteGlu binding activity†: Purification, -fold	Ratio of activities: Binding/dimethylglycine	Ratio of activities: Binding/sarcosine
Mitochondrial extract	4.5	327.0	0.65	2.55	39	100	1	60	15
Sephadex G-150 chromatography	22.0	75.9	2.49	28.8	197	117	5	79	7
DEAE-Cellulose chromatography	13.7	25.2	14.2	4.85‡	542	107	14	38	112‡
Affinity chromatography	6.6	2.61	82.3	22.5	2520	52	65	31	112

* One unit is equivalent to 1 μmol of substrate oxidized per min at 25°C. The dehydrogenase activity of the mitochondrial extract was assayed in the absence of phenazine methosulfate; endogenous electron-transferring flavoprotein (11) was the intermediate electron carrier.

† One picomole of binding activity is equivalent to 44 dpm of H_4[^{3}H]PteGlu (20 μCi/μmol). Activity was not corrected for losses during the assay procedure.

‡ The large decrease in sarcosine dehydrogenase activity at this point was due to separation of another protein having this activity (see text).

column and was not eluted even when a gradient running from 0 to 1.0 M KCl was applied (Fig. 1). At this point a second gradient of 0–20 mM PteGlu in 1 M KCl was applied and a peak of protein was eluted from the column. This peak of protein corresponded to the folate-binding activity and to the activity of dimethylglycine dehydrogenase (also shown in Fig. 1).

The purification of this protein is summarized in Table 1. Both the folate-binding activity and the dimethylglycine dehydrogenase activity were purified in parallel in the last two steps of the procedure. The dehydrogenase activity of the protein with sarcosine as substrate was about 25% that seen with dimethylglycine. Sarcosine dehydrogenase activity also copurified with folate-binding activity in the last two purification steps.

The large loss of sarcosine dehydrogenase activity after DEAE-cellulose chromatography was due to the separation of another protein having this activity. This separate sarcosine dehydrogenase activity coeluted with the minor peak of folate-binding activity noted above, suggesting the presence of a second folate-binding protein having dehydrogenase activity specific for sarcosine. This protein was not further characterized in the present study.

Fig. 2 shows a densitometric scan of a $NaDodSO_4$ gel, stained with Coomassie blue, prepared from the material obtained after affinity chromatography. Only a single band was present. By comparison with standard compounds run simultaneously, the molecular weight is about 90,000.

The purified protein was yellow and had an absorption spectrum consistent with it being a flavoprotein (Fig. 3). The two peaks appearing at about 450 and 360 nm are characteristic of flavins (12). Moreover, the persistence of this spectrum in a sample treated with urea and $NaDodSO_4$ and subjected to electrophoresis is strong evidence that the flavin is tightly bound to the protein.

The absorption spectrum of the native protein (Fig. 4) had maxima at 345 and 457 nm. The shoulders around 435 and 480 nm are characteristic of many flavoproteins (12). The large absorbance at 345 is probably due in part to protein-bound PteGlu which was used to elute the protein from the affinity column and which has an absorption maximum at 346 nm (3). As expected, dimethylglycine and sarcosine (250 molar excess) were effective in bleaching the 457 nm absorbance. Dimethylglycine and dithionite had similar effects on the protein spectrum.

DISCUSSION

Dimethylglycine dehydrogenase and sarcosine dehydrogenase were partially purified from rat liver mitochondria and separated by Frisell and Mackenzie in 1962 (11). At that time, a 14-fold purification of dimethylglycine dehydrogenase was achieved by using $(NH_4)_2SO_4$ precipitation, DEAE-cellulose chromatography, and calcium phosphate gel separation. Their work suggested that both enzymes were flavoproteins which interacted with the electron transfer flavoprotein in crude extracts but required phenazine methosulfate as primary electron acceptor in partially purified preparations. The molecular

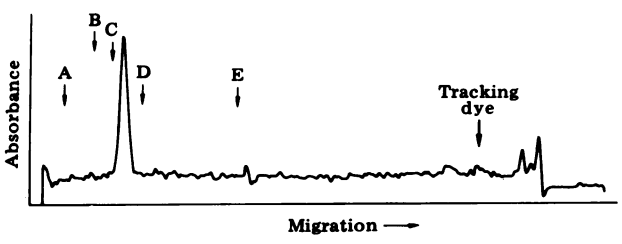

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified mitochondrial folate-binding protein. Protein (10 μg) was treated with 1% $NaDodSO_4$ and 1% 2-mercaptoethanol, heated 5 min in a boiling water bath, and electrophoresed in a 0.15 × 10 × 14 cm slab gel. After staining with Coomassie blue, the gel was dried onto a cellulose acetate sheet (Bio-Rad model 224 gel slab drier) and scanned (Corning model 740 densitometer) at 550 nm. The arrows indicate the positions to which the marker proteins migrated: A, myosin; B, β-galactosidase; C, phosphorylase B; D, bovine serum albumin; E, ovalbumin.

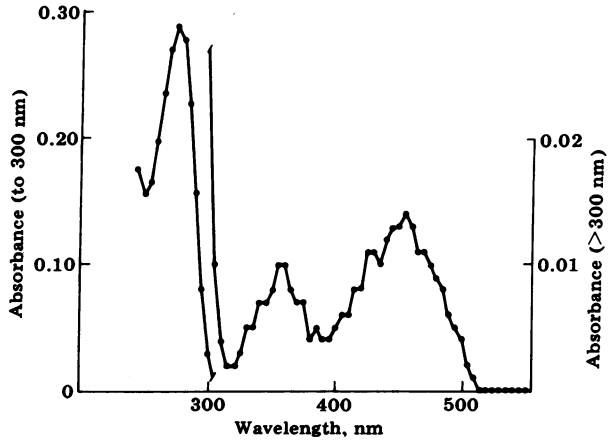

FIG. 3. Absorption spectrum of purified mitochondrial folate-binding protein after urea treatment and $NaDodSO_4$/polyacrylamide gel electrophoresis. Purified protein (42 μg) was made 6 M in urea and 1.5% in $NaDodSO_4$, heated in a boiling water bath for 5 min, and subjected to electrophoresis in a 9 × 0.5 cm cylindrical gel. The spectrum of the protein in the unstained gel was obtained with a Gilford spectrophotometer equipped with a linear transport. Absorbance was read at various wavelengths both at the protein peak and at an area of the gel devoid of protein and the difference was calculated.

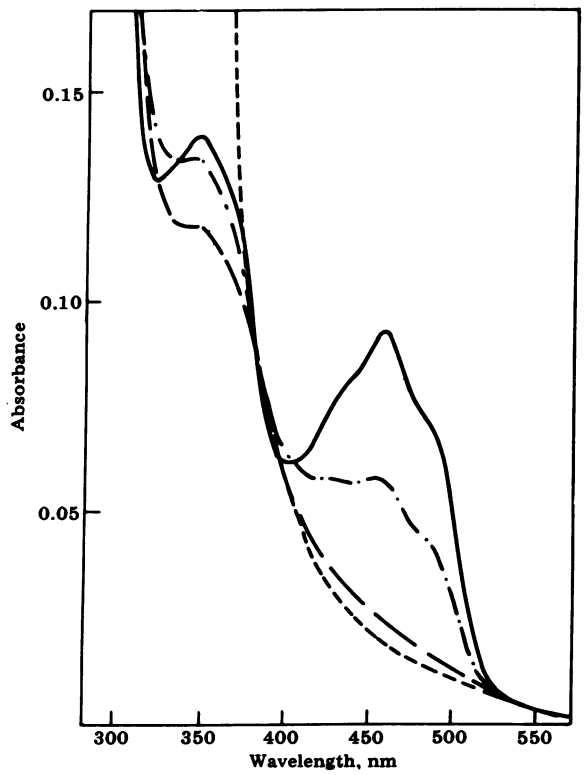

FIG. 4. Absorption spectra of purified mitochondrial folate-binding protein. —, Protein as isolated; - - -, protein plus 200 nmol of sarcosine; – –, protein plus 200 nmol of dimethylglycine; - - -, protein plus a few grains (about 0.1 mg) of sodium dithionite. Before each spectrum was recorded, the absorbance at 600 nm was arbitrarily set at zero to eliminate instrument fluctuation and facilitate comparison of the spectra. Only small adjustments (0.02 absorbance unit or less) were required. The absorbance of the protein as isolated was 0.014 at 600 nm. The protein concentration was 0.72 mg/ml and the volume in the cuvette was 100 μl. Spectra were obtained with a Cary 219 spectrophotometer. The protein had been dialyzed extensively against 10 mM potassium phosphate (pH 7.8).

weights of these proteins were not measured, but an estimate of about 300,000 was made on the basis of the flavin content (assuming 1 mol of flavin per mol of protein). Hoskins and Bjur (6) described the purification of sarcosine dehydrogenase from liver mitochondria of the monkey. Again, no molecular weight was calculated but a sedimentation coefficient of 4.3 S was obtained. Assuming a normal globular protein, this is equivalent to 60,000–65,000.

More recently, groups working in Japan (13) and the United States (14) have investigated the nature of the proteins of mitochondria that contain covalently bound flavin. Both have used the technique of labeling these proteins by injection of [^{14}C]riboflavin into rats and by separation of the covalently labeled proteins from the mitochondria. NaDodSO$_4$/polyacrylamide gel electrophoresis showed only four proteins that retained the bound ^{14}C. Both groups estimated the molecular weight of one of these proteins to be 91,000 by this technique (13, 14). Subsequently, Sato *et al.* (15) reported a partial purification of this protein. When electrophoresis was carried out in the absence of denaturing conditions followed by incubation in a mixture containing phenazine methosulfate and 2,3,4-triphenyltetrazolium, the major band was identified as having sarcosine dehydrogenase activity. This band also displayed greater activity with dimethylglycine as substrate.

We believe that the purified folate-binding protein from mitochondria is the same as the one that has been identified by Sato *et al.* (15) as sarcosine dehydrogenase for the following reasons. First, the folate binding protein from mitochondria is a flavoprotein which contains tightly bound flavin. Second, the estimated size of the protein is 90,000 daltons, both by NaDodSO$_4$ electrophoresis and by gel filtration (1). Third, the final preparation contains only a single protein. Any contaminant must be present only in trace quantities. Because dimethylglycine is effective in producing spectral changes in the protein consistent with complete reduction of the flavin moiety, it is highly unlikely that the dehydrogenase activity is due to a contaminating protein. Similarly, because the purified protein has a 1:1 stoichiometry for folate binding (unpublished data), this activity cannot be due to a minor impurity. Fourth, it is highly unlikely that a contaminating protein would selectively adsorb to an affinity column bearing 5-formyltetrahydrofolic acid as the ligand and remain bound in the presence of 1 M KCl only to be eluted by PteGlu, coincident with the H$_4$PteGlu binding activity. Fifth, the reaction catalyzed by dimethylglycine dehydrogenase is particularly suited for the participation of H$_4$PteGlu as a cofactor.

Fig. 5 shows a reaction sequence for the dimethylglycine and sarcosine dehydrogenases. As usually depicted, the one-carbon product is formaldehyde or "active formaldehyde" as termed by Mackenzie (16). The exact nature of the active formaldehyde in these reactions has remained unknown, but recent studies by Frisell and coworkers (17) have strongly implicated 5,10-methylenetetrahydrofolate. The spontaneous reaction of H$_4$PteGlu and formaldehyde to form 5,10-methylenetetrahydrofolate is well known (3). The fact that this enzyme binds H$_4$PteGlu *in vitro* and can be isolated containing bound H$_4$PteGlu$_5$ suggests that it may function as a prosthetic group. Subsequent loss of the formaldehyde to regenerate the active enzyme may take place by transfer of the one-carbon unit by itself or by dissociation of the 5,10-methylenetetrahydrofolate polyglutamate from the enzyme.

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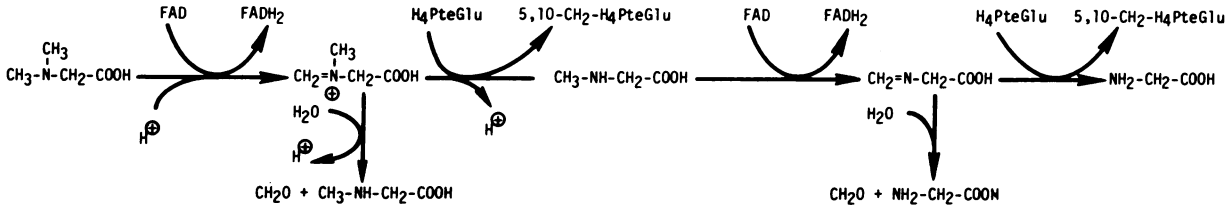

FIG. 5. Proposed involvement of H$_4$PteGlu in the oxidation of dimethylglycine to glycine.

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