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PURIFICATION OF GLYCINEAMIDE RIBONUCLEOTIDE TRANSFORMYLASE*

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SUMMARY

Glycineamide ribonucleotide transformylase has been purified 240-fold from chicken liver along with formyl-methenyl-methylenetetrahydrofolate synthetase (combined), through seven purification steps including a glycineamide ribonucleotide affinity column and a 10-formyl synthetase specific MgATP elution from hydroxylapatite. The transformylase is a homodimer of M_r 63,000 whereas the synthetase (combined) is composed of two identical subunits of M_r 83,000. Transformylase has been isolated free of the synthetase (combined) by an additional anion exchange chromatographic step resulting in a net 400-fold purification.

Our interest in the chemistry of tetrahydrofolic acid derivatives (1-5) prompted an investigation of glycineamide ribonucleotide transformylase (5,10-methenyltetrahydrofolate: 2-amino-N-ribosylacetamide-5'-phosphate formyltransferase, E.C.2.1.2.2), an enzyme in the purine biosynthetic pathway which utilizes 5,10-methenyltetrahydrofolate as a cofactor for the transfer of a one-carbon unit at the formate level of oxidation. Purification of the transformylase from chicken liver resulted in concommitant enrichment of 10-formyltetrahydrofolate synthetase, 5,10-methenyltetrahydrofolate cyclohydrolase, and 5,10-methylenetetrahydrofolate dehydrogenase as demonstrated in this communication.

Warren and Buchanan (6) purified the transformylase 56-fold from avian liver and noted that their preparation exhibited cyclohydrolase activity. No further reports on the avian liver enzyme have appeared.

*Glycineamide ribonucleotide transformylase (GAR-transformylase) is the trivial name for 5,10-methenyltetrahydrofolate: 2-amino-N-ribosylacetamide-5'-phosphate formyltransferase, (EC.2.1.2.2).

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Abbreviations: GAR, Glycineamide ribonucleotide; DS, sodium dodecyl sulfate; 2-met, 2-mercaptoethanol; DEAE-, diethyl-amino-ethyl.

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MATERIALS AND METHODS

Materials. Bio-Gel HTP (hydroxylapatite) was obtained from Bio-Rad. Sephadex G-100, G-25 (coarse) and CNBr-activated Sepharose 4B were obtained from Pharmacia. Whatman CF-1 Cellulose and DEAE-Cellulose (DE-52) were purchased from Reeve-Angel. Type II-O ovomucoid, α-1-antitrypsin, Aprotinin, protamine sulfate, tetrahydrofolic acid, phosphorylase-a, bovine serum albumin, fumarase, yeast alcohol dehydrogenase and catalase were obtained from Sigma. L-(+)-5,10-methenyltetrahydrofolic acid was prepared as described by Rowe (7). Glycineamide ribonucleotide was prepared as described previously (8). CAR-Sepharose was prepared according to Chettur (9).

Gel electrophoresis. Disc gel electrophoresis was performed according to the method of Davis (10) and dodecyl sulfate gel electrophoresis as described by Weber et al. (11).

Sucrose density gradient ultracentrifugation. The method of Martin and Ames (12) was utilized and centrifugation was performed with an SW-27 rotor at 25,000 rpm on a Beckman L3-50 Ultracentrifuge.

Enzyme assays. The assay for glycineamide ribonucleotide transformylase was based on the production of tetrahydrofolic acid as determined by the Bratton-Marshall (13) assay for diazotizable amine described previously (14). The incubation mixture (37°) contained 25 mM maleate, pH 6.8; 0.1 mM EDTA: 0.28 mM glycineamide ribonucleotide, and enzyme in a volume of 0.8 ml. After 10 min., 5,10-methenyltetrahydrofolic acid was added to give a 0.12 mM solution. The reaction was quenched with 0.1 ml of 30% trichloroacetic acid ten minutes after the addition of 5,10-methenyltetrahydrofolic acid. 0.75 ml of the reaction solution was treated with 0.1 ml of 1N H_2SO_4, 0.05 ml of 0.1% sodium nitrite, 0.05 ml of 0.5% ammonium sulfamate and 0.05 ml of 0.1% N-napthylethylene diamine dihydrochloride in 0.01 N HCl. The absorbance at 540 nm was measured after 30 minutes.

Cyclohydrolase activity was assayed by following the disappearance of 5,10-methenyltetrahydrofolic acid monitored at 355 nm (15). The cuvette contained 120 mM potassium maleate, pH 7.2; 200 mM 2-mercaptoethanol and 0.06 mM 5,10-methenyltetrahydrofolic acid at 35°. After the rate of non-enzymic hydrolysis had been established, enzyme was added and the rates were obtained with a thermostated Gilford recording spectrophotometer.

Synthetase was assayed (16) at 37° in an incubation mixture containing 75 mM potassium maleate, pH 7.2; 5 mM ATP, 40 mM sodium formate, 10 mM $MgCl_2$; 100 mM NH_4Cl, 1 mM 1-(+)-tetrahydrofolic acid, 100 mM 2-mercaptoethanol and enzyme in a volume of 0.9 ml. The reaction was terminated after 10 min. with 0.1 ml of 6N HCl. The 10-formyltetrahydrofolic acid was measured as the 5,10-methenyl derivative at 350 nm after acidification.

Dehydrogenase was assayed by monitoring the production of NADPH at 340 nm (17). The cuvette contained 40 mM potassium phosphate, pH 7.5; 0.06 mM formaldehyde; 0.25 mM L-(+)-tetrahydrofolic acid; 25 mM 2-mercaptoethanol; 0.25 mM $NADP^+$ and enzyme in a volume of 1 ml at 35°.

Protein was determined by assuming an extinction of 1.0 for a 1 mg/ml solution or by the Lowry method (18) using bovine serum albumin as standard.

Enzyme purification.

Extract. Fresh chicken liver, obtained from the Poultry Plant, Pennsylvania State University immediately after death of the animal, was chilled on ice and used immediately. The liver (150 g) was homogenized with four volumes of 0.01 M potassium phosphate, pH 7.5-10 mM 2-met-1 mM EDTA containing 250 mg of Type II-O ovomucoid, 25 mg of α-1-antitrypsin and 2.5 ml of Aprotinin per liter in an Oster Blender. The homogenate was centrifuged at 18,000 x g for 35 min. and the supernatant was filtered through cheese cloth.

Protamine sulfate fractionation. 150 ml of 2% protamine sulfate in 0.01 M potassium phosphate, pH 7.5 was added slowly to the stirred supernatant while the pH was maintained at pH 7.5 with 4N NH_4OH. After 20 minutes, the suspension was centrifuged at 18,000 xg for 10 minutes.

Ammonium sulfate fractionation. The supernatant was brought to 40% saturation by addition of solid $(NH_4)_2SO_4$ while the pH was maintained at pH 7.5 with NH_4OH. After one hour, the suspension was centrifuged at 18,000 xg for 10 minutes. The supernatant was brought to 55% saturation with $(NH_4)_2SO_4$ as above. The 40-55% pellet was collected by centrifugation and dissolved to 250 ml with homogenization buffer.

Bio-Gel HTP chromatography. The protein was applied to a 6 x 8.6 cm column made from 30 g Bio-Gel HTP and 30 g of Whatman Cellulose CF-1 and equilibrated with 0.01 M potassium phosphate, pH 7.5-10 mM 2-met. The column was washed with 600 ml of 0.01 potassium phosphate, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v), then with 1 l of 0.033 M potassium phosphate, pH 7.5 - 10 mM 2-met - 25% glycerol (V/v) and the protein was then eluted with 0.1 M potassium phosphate, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v). The transformylase containing fractions were combined and concentrated 8-fold on a Amicon Ultrafiltration unit with a UM-20 membrane.

Sephadex G-100 chromatography. The concentrated protein was applied to a 3 x 100 cm column of Sephadex G-100 equilibrated with 0.05 M potassium phosphate, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v). The column was developed with the same buffer. The transformylase containing fractions were combined and dialyzed against 20 volumes of 0.01 Tris·HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v).

Bio-Gel HTP chromatography. 75% of the G-100 fraction was applied to a 4 x 7 cm column made from 8 g of Bio-Gel HTP and 8 g of Whatman Cellulose CF-1 that had been equilibrated with 0.01 Tris·HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v). The column was washed with 200 ml of starting buffer and then with 300 ml of 0.1 M Tris HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v). Transformylase was eluted with 200 ml of 0.1 M Tris HCl, pH 7.5 - 10 mM 2-met 25% glycerol (v/v) containing 5 mM ATP/5 mM $MgCl_2$ and concentrated to 30 ml with an Amicon Ultrafiltration unit employing an XM-50 membrane. ATP was separated from the protein by desalting on a 2.5 x 30 cm column of Sephadex G-25 (coarse) that had been equilibrated with 0.05 M potassium phosphate, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v) and elution with this buffer.

GAR-Sepharose chromatography. A small portion of the desalted protein was dialyzed against 130 volumes of 0.01 M Tris HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v) and applied to a 1.4 x 8.5 cm column of GAR-Sepharose that had been equilibrated with the same buffer. The column was washed with 40 ml of starting buffer, then with 40 ml of 0.1 M Tris HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v) and transformylase was eluted with 0.02 M potassium phosphate, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v). The transformylase containing fractions were combined and concentrated to 2 ml with an Amicon Ultrafiltration unit equipped with an XM-50 membrane.

DEAE-Cellulose chromatography. As an alternate procedure, a portion (25%) of the Sephadex G-100 fraction was dialyzed against 75 volumes of 0.001 M potassium phosphate, pH 7.8 - 10 mM 2-met - 25% glycerol (v/v) and applied to a 2.5 x 17 cm column of DEAE-Cellulose that had been prepared according to the manufacturer's directions and equilibrated with dialysis buffer. The column was washed with 500 ml of starting buffer and then eluted with a gradient made from 500 ml of 0.001 M potassium phosphate, pH 7.8 - 10 mM 2-met - 25% glycerol (v/v) and 500 ml of 0.005 M potassium phosphate, pH 7.8 - 10 mM 2-met - 25% glycerol (v/v). The highest specific activity transformylase fractions were combined and concentrated 6 fold with an Amicon Ultrafiltration unit equipped with an XM-50 membrane.

GAR-Sepharose chromatography. One milliliter of the DEAE-Cellulose fraction was dialyzed against 1 l of 0.01 M Tris HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v) and applied to an 1.4 x 8.5 cm column of GAR-Sepharose equilibrated with the dialysis buffer. The column was washed with 30 ml of starting buffer, then with 40 ml of 0.1 M Tris HCl, pH 7.5 - 10 mM 2-met - 25% glycerol (v/v) and the transformylase was eluted with 0.1 M Tris HCl, pH 7.5 - 10 mM 2-met - 25% gylcerol (v/v) containing 0.01 M potassium phosphate, pH 7.5. The transformylase fractions were combined and concentrated 6 fold with an Amicon Ultrafiltration unit employing an XM-50 membrane.

Table I

Purification of GAR Transformylase

Purification Step	Volume (ml)	Total Protein (g)	Units[a]	Units/mg	Purification	(yield)
1. Homogenate supernatant	400	27.7	87.5	0.0032	1.0	(100)
2. Protamine sulfate supernatant	500	13.5	88.5	0.0065	2.0	(100)
3. Ammonium sulfate 40-55%	250	7.2	81.5	0.0113	3.5	(92)
4. Bio-Gel HTP	55	1.463	61.1	0.0417	13.0	(69)
5. Sephadex G-100[b]	110	0.6435	30.1	0.0465	14.3	(34)
6. Bio-Gel HTP (MgATP)[c]	40	0.2264	22.2	0.0981	31.0	(25)
7. GAR-Sepharose[d]	2	0.000828	0.63	0.7616	238.0	
6a. DEAE-Cellulose[e]	4	0.0246	3.59	0.146	45.6	
7a. GAR-Sepharose[f]	8	0.000068	0.088	1.294	404.0	

a) Units are expressed as micromoles per minute.
b) Approximately 50% of the total protein was removed by this step. The specific activity (hence, the yield) is low due to high phosphate inhibition of the transformylase assay (24).
c) 75% of the G-100 fraction was applied to the HTP column, hence the yield of this column is 98%.
d) A small portion of the HTP fraction was chromatographed on GAR-Sepharose.
e) 25% of the G-100 fraction was chromatographed on DEAE-Cellulose.
f) A small portion of the DEAE-Cellulose fraction was applied to the GAR-Sepharose.

RESULTS AND DISCUSSION

Enzyme Purification. The purification of the transformylase is summarized in Table I. The ratios of the specific activities for the four folate enzymes during the course of transformylase purification are outlined in Table II. The four activities, designated as A, B, C and D were initially in the ratios 1:9.2:6.7:2.1 and remained essentially constant through the purification step employing ATP (a substrate only for the synthetase) elution of the second Bio-Gel HTP column. The ratios of the dehydrogenase and the cyclohydrolase to the synthetase activity are 0.2 and 0.7 which are similar to those for the trifunctional protein isolated from sheep liver (0.2 and 0.5, respectively) (19) and porcine liver (0.5 and 1.6, respectively) (20). Selective purification of the transformylase activity is achieved by chromatography on a glycineamide ribonucleotide affinity column altering the A:B:C:D ratios to 1:0.84:1.04:1.03 and effecting a 240-fold purification of the transformylase. Although the protein gives a single band on analytical gel electrophoresis, in the presence

Table II

Specific Activity Ratios for the Folate Activities During Purification of GAR-Transformylase

Purification Step	Specific Activity Ratios[a]		
	B/A	C/A	D/A
1. Homogenate supernatant	9.2	6.7	2.1
2. Protamine sulfate supernatant	8.6	3.9	2.6
3. Ammonium sulfate 40-55%	16.2[b]	4.8	2.6
4. Bio-Gel HTP	9.0	3.7	2.4
5. Sephadex G-100	19.9[b]	4.9	2.2
6. Bio-Gel HTP (MgATP)	6.4	3.6	2.1
7. GAR-Sepharose	0.84	1.04	1.03
6a. DEAE-Cellulose	0.26	0.48	0.12
7a. GAR-Sepharose	0	0	0

[a]The four folate activities are: A, glycineamide ribonucleotide transformylase; B, 10-formyltetrahydrofolate synthetase; C, 5,10-methenyltetrahydrofolate cyclohydrolase; and D, 5,10-methylenetetrahydrofolate dehydrogenase.

[b]10-formyltetrahydrofolate synthetase activity is enhanced by NH_4^+ and K^+ (25).

of DS two bands with relative intensities of 1.7:1 are detected. Resolution of the transformylase from the other three activities (B, C and D) has been achieved by employing chromatography on DEAE-Cellulose as shown in Fig. 1, followed by GAR-Sepharose chromatography. This transformylase fraction is essentially homogeneous by the criterion of DS gel electrophoresis, exhibiting a single band. As in the case of the B, C and D activities isolated from sheep (19) and porcine (20) liver and from yeast (21), a single trifunctional

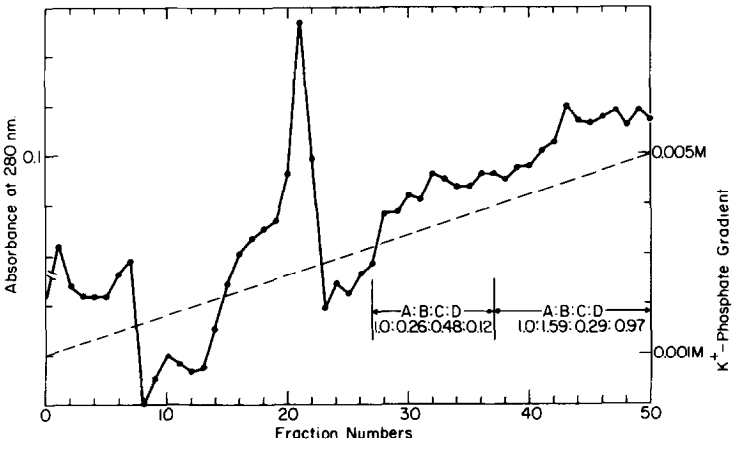

Figure 1. Protein profile from DEAE-cellulose column developed with a gradient of potassium phosphate as described in 'Methods'; A, glycineamide ribonucleotide transformylase; B, 10-formyltetrahydrofolate synthetase; C, 5,10-methenyltetrahydrofolate cyclohydrolase; D, 5,10-methylenetetrahydrofolate dehydrogenase.

protein, designated formyl-methenyl-methylenetetrahydrofolate synthetase (combined), appears to be responsible for these three catalytic activities.

Native and Subunit Molecular Weight. The molecular weight of the protein possessing transformylase activity has been estimated from sucrose density gradient ultracentrifugation (12) as 100,000 and that for the trifunctional protein (which is partially resolved from the transformylase by this technique) as 160,000. Molecular weights of 201,000, 218,000 and 150,000 have been assigned to the trifunctional protein isolated from sheep liver (19), porcine liver (20) and the yeast (21), respectively, as determined by gel filtration. The subunit molecular weight of transformylase has been estimated as 63,000 and that of the trifunctional protein as 83,000 from their relative mobilities on DS gel electrophoresis (11). Thus both proteins apparently have molecular weights consistent with homodimeric structures. The trifunctional protein from sheep liver (19) likewise consists of two identical subunits, whereas the native structure from porcine liver (20) is probably a single subunit.

Discussion. The co-purification of formyl-methenyl-methylenetetrahydrofolate synthetase (combined) and glycineamide ribonucleotide transformylase

raises the possibility that the two proteins may specifically aggregate or be covalently linked in the native state. Although the purification sequence was designed to minimize the exposure of the protein to proteolysis by liver proteases during isolation (rapid total protease removal was monitored by a ^{14}C-hemoglobin assay) (22), the possibility remains that all the activities may be found on a single polypeptide. Proteolytic processing of the dimeric, trifunctional protein from yeast results in the formation of a dimeric, monofunctional formyltetrahydrofolate synthetase (21). Proteolytic degradation is particularly troublesome in chicken livers as illustrated by the difficulties encountered in the isolation of fatty acid synthetase from this source (23).

Evidence for the noncovalent interaction of the trifunctional protein with the transformylase is based on i) their co-elution from Bio-Gel HTP with MgATP, a reverse affinity step previously used in the purification of the sheep liver synthetase (combined) enzyme (19), ii) their co-elution from a GAR-Sepharose affinity column, although transformylase is enhanced, iii) recombination experiments of fractions possessing predominantly synthetase activity with those enhanced in transformylase activity that yield an increase in the specific activity of transformylase up to 2 fold (24), and iv) the decrease of the trifunctional protein activities relative to transformylase activity upon separation of these proteins. The association of enzymes which catalyze 5,10-methenyltetrahydrofolate synthesis and require this cofactor for a one carbon unit transfer offers a solution for the efficient utilization of the hydrolytically labile 5,10-methenyl species.

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