inhardt, T. A., & Horst, R. L. (1981b) *Helv. Chim. Acta 64*, 2138.
Partridge, J. J., Chadha, N. K., Shiuey, S.-J., Wovkulich, P., Uskokovic, M. R., Napoli, J. L., & Horst, R. L. (1982) in *Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism* (Norman, A. W., Schaefer, K., Herrath, D.v., Grigoleit, H.-G., Eds.) pp 1073–1078, Walter de Gruyter, Berlin.
Reinhardt, T. A., Napoli, J. L., Pramanik, B. C., Littledike, E. T., Beitz, D. C., Partridge, J. J., Uskokovic, M. R., & Horst, R. L. (1981) *Biochemistry 20*, 6230.
Reinhardt, T. A., Napoli, J. L., Beitz, D. C., Littledike, E. T., & Horst, R. L. (1982) *Arch. Biochem. Biophys. 213*, 163.
Reinhardt, T. A., Horst, R. L., Orf, J., & Hollis, B. W. (1984) *J. Clin. Endocrinol. Metab. 58*, 91.
Tanaka, Y., Wichmann, J. K., Parren, H. E., Schnoes, H. K., & DeLuca, H. F (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6411.
Wichmann, J. K., DeLuca, H. F., Schnoes, H. K., Horst, R. L., Shepard, R. M., & Jorgensen, N. A. (1979) *Biochemistry 18* 4775.

An Antibody Probe To Determine the Native Species of Glycinamide Ribonucleotide Transformylase in Chicken Liver†

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ABSTRACT: Antibody probes of Western blots [Renart, J., Reiser, J., & Stark, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3116] of chicken liver homogenates under various conditions revealed that glycinamide ribonucleotide transformylase can be rapidly proteolyzed in such homogenates. These findings, along with molecular weight measurements by ultracentrifugation, identify the true form of glycinamide ribonucleotide transformylase as a monomeric protein of 117 000 daltons. This protein has been purified 400-fold in 44% yield from chicken liver in one step on an affinity column of 10-formyl-5,8-dideazafolate–Sepharose. Native glycinamide ribonucleotide transformylase retains full activity after proteolytic cleavage to a form (M_r 55 000) similar to fragments seen in the Western blot of the homogenates. This phenomenon may be responsible for the previous identification of glycinamide ribonucleotide (GAR) transformylase as a dimer of 55 000-dalton subunits. Similar analyses using antibodies to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase [Mueller, W. T., & Benkovic, S. J. (1981) *Biochemistry 20*, 337] and trifunctional enzyme [Smith, G. K., Mueller, W. T., Wasserman, G. F., Taylor, W. D., & Benkovic, S. J. (1980) *Biochemistry 19*, 4313] confirm that these two proteins were isolated in their native forms.

Glycinamide ribonucleotide transformylase (GAR transformylase, EC 2.1.2.2)[1] catalyzes the transfer of a formyl group from (6*R*)-10-CHO-H_4folate to glycinamide ribonucleotide and is one of two such reduced-folate-requiring enzymes in the purine biosynthetic pathway. This enzyme has been purified previously by Warren & Buchanan (1957) and subsequently by workers in this laboratory (Caperelli et al., 1978, 1980). The latter group associated the transformylase activity with a homodimer protein of 55 000-dalton subunits on the basis of the results of sucrose density ultracentrifugation and SDS–polyacrylamide gel electrophoresis studies. Smith et al. (1980) have shown that GAR transformylase activity copurifies through several steps with another enzyme: the 5,10-methylene-, 5,10-methenyl-, and 10-formyl-H_4folate synthetase (combined) (trifunctional enzyme, EC 6.3.4.3, EC 1.5.1.5, and EC 3.5.4.9), which catalyzes interconversions between reduced one-carbon-substituted folate cofactors.

In this paper we report that GAR transformylase is a single-subunit protein of about 117 000 daltons in chicken liver. Evidence in support of this conclusion comes primarily from Western blot analysis of trichloroacetic acid homogenized liver extracts. Probes of the same blots with antibodies to AICAR transformylase (EC 2.1.2.3; Mueller & Benkovic, 1981) and trifunctional enzyme indicate that the native subunit molecular weight of these proteins is 67 000 and 95 000, respectively. These subunit molecular weights are in good agreement with those obtained previously. The 117 000-dalton protein has been purified to near homogeneity in a single chromatographic step on a column of 10-formyl-5,8-dideazafolate–Sepharose, which has been employed previously in the purification of thymidylate synthetase from L1210 mouse leukemia cells by Rode et al. (1979). We present evidence for a limited in vitro proteolysis to the 55 000-dalton form that retains full activity, indicating that this cleavage may have been responsible for the previous identification of GAR transformylase as a dimer.

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[1] Abbreviations: GAR, α,β-glycinamide ribonucleotide; H_4folate, tetrahydrofolate; 5,10-C^+H-H_4folate, (6*R*)-5,10-methenyltetrahydrofolate; 10-CHO-H_4folate, (6*R*)-10-formyltetrahydrofolate; 2-ME, 2-mercaptoethanol; GAR transformylase, (6*R*)-10-formyltetrahydrofolate:5′-phosphoribosylglycinamide formyltransferase; trifunctional enzyme, 5,10-methenyltetrahydrofolate, 5,10-methylenetetrahydrofolate, and 10-formyltetrahydrofolate synthetase (combined); SDS, sodium dodecyl sulfate; NP-40, Nonidet P-40; FGAR, N^β-formylglycinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide 1-ribonucleotide; AICAR transformylase, (6*R*)-10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase; anti-GAR, antiserum to GAR transformylase; anti-AICAR, antiserum to AICAR transformylase; anti-TP, antiserum to trifunctional protein; TCA, trichloroacetic acid; APT, (aminothio)phenol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

0006-2960/84/0423-3979$01.50/0

Experimental Procedures

Materials

Folic acid, yeast alcohol dehydrogenase, pig heart malate dehydrogenase, SDS gel molecular weight marker proteins, pepstatin A, α-1-antitrypsin, type II-O ovomucoid trypsin inhibitor, and aprotinin were purchased from Sigma Chemical Co., St. Louis, MO. Sepharose 4B and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. IgGsorb was obtained from The Enzyme Center, Inc., Boston, MA. Freund's complete adjuvant is the product of Difco Laboratories, Detroit, MI. Kodak XAR-5 film and Du Pont Cronex Lightning-Plus intensifier screens were acquired from Picker International, Highland Heights, OH. ^{125}I-Labeled protein A and [^{14}C]formaldehyde were purchased from New England Nuclear, Boston, MA. All other chemicals were obtained from either Sigma Chemical Co., St. Louis, MO, Fisher Scientific Co., Fairlawn, NJ, or Aldrich Chemical Co., Milwaukee, WI, and were used without further purification.

GAR was synthesized by the method of Chettur & Benkovic (1977). (6*RS*)-H_4folate was synthesized by reduction of folic acid with (dimethylamino)borane according to the method of Martinelli & Chaykovsky (1980), with the modification that the reduction was carried out under dry nitrogen. Dihydrofolate was produced from folic acid by the method of Blakeley (1960). Enzymatic reduction of dihydrofolate in the manner of Mathews & Huennekens (1960) produced (6*S*)-H_4folate. (6*RS*)-H_4folate and (6*S*)-H_4folate were converted to (6*RS*)-5,10-C$^+$H-H_4folate and (6*R*)-5,10-C$^+$H-H_4folate, respectively, by the method of Rowe (1968).

5,8-Dideazafolate was synthesized by a modification of the method of Acharya & Hynes (1975). 2-Amino-6-(bromomethyl)-4-hydroxyquinazoline (1.792 g) was stirred with diethyl (4-aminobenzoyl)-L-glutamate (2.42 g) at room temperature for 3 days in 40 mL of dimethylacetamide. Solvent was removed on a vacuum rotary evaporator, and then water was added to the residue. The slurry was brought to pH 3.5 with 6 N HCl. After being allowed to stand on ice 1 h, the mixture was decanted, and the residue was dissolved in 125 mL of 0.25 M KOH and 20 mL of dimethylacetamide. The solution was stirred at room temperature for 24 h. After the pH was adjusted to 8 with 1 N HCl, the solution was applied to a 4 × 50 cm DEAE-Sephadex A-25 column at 4 °C equilibrated with 0.1 M triethylammonium bicarbonate (TEAB). Fractions of 21 mL were collected. Product was eluted with a linear gradient formed from 2 L of 0.1 M TEAB and 2 L of 2.0 M TEAB and appeared in fractions 150–182. 10-Formyl-5,8-dideazafolate was prepared by formylation of 5,8-dideazafolate according to Smith et al. (1981a). (Aminothio)phenol (APT) paper was prepared by the procedure of Seed (1982).

Methods

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed on 8.75% slab gels as described by Laemmli (1970) or by Neville (1971) with the following proteins as molecular weight markers: carbonic anhydrase (M_r 29 000), albumin (egg) (M_r 45 000), bovine serum albumin (M_r 66 000), and rabbit muscle phosphorylase B (M_r 97 400). The gels were stained and destained by the method of Weber et al. (1972).

Synthesis of 10-Formyl-5,8-dideazafolate–Sepharose. The affinity column was prepared by the general procedure of Cuatrecasas & Anfinsen (1971) with the following variations. A total of 100 mL of Sepharose 4B was washed with 10 successive 100-mL portions of water by suction filtration, taking care not to dry the resin. The moist resin was transferred to an ice-cooled beaker equipped with a Teflon stir bar and pH electrode in a fume hood and suspended in 50 mL of 0.25 M potassium phosphate, pH 10.5. A solution of 16 g of BrCN in 25 mL of dioxane was added dropwise to the stirring suspension over 25 min. The pH of the reaction was kept at 10.5–11.5, and its temperature was not allowed to exceed 10 °C. Reaction continued for an additional 15 min, after which the pH had stabilized. The reaction mixture was vacuum filtered into a flask containing 50 g of KOH in 200 mL of H_2O, again taking care not to let the resin dry. The activated Sepharose was washed with 6 times 200 mL of H_2O and then transferred to a stoppered flask, and 25 mL of ethylenediamine was added. The flask was cooled on an ice bath, sealed, and agitated at 25 °C for 8 h. The ethylenediamine–Sepharose was filtered and washed with 4 times 250 mL of 1 M NaCl, followed by 4 times 250 mL of H_2O. The moist resin was suspended in a minimum volume of H_2O.

A solution of 275 μmol of 10-formyl-5,8-dideazafolate in 30 mL of 50% (v/v) dimethylacetamide–water, pH 8.0, was added to the suspension of ethylenediamine–Sepharose in water. A total of 2.0 g of *N*-ethyl-*N*-[3-(dimethylamino)-propyl]carbodiimide hydrochloride was added directly to the mixture, and the pH was adjusted to 5.6. The reaction mixture was shaken in a sealed flask for 10 h at 25 °C. A second addition of 0.5 g of carbodiimide was made, and the reaction was allowed to continue for 10 h more at pH 5.6. The mixture was filtered, and the resin was washed 10 times with the following sequence: 200 mL of 0.1 M $NaHCO_3$–0.5 M NaCl, pH 9.6, then 200 mL of H_2O, and then 200 mL of 0.2 M sodium acetate–0.5 M NaCl, pH 4.6.

The mother filtrate was shown to be devoid of 5,8-dideazafolate or 10-formyl-5,8-dideazafolate by UV absorbance and GAR transformylase enzymatic assay, respectively. The affinity resin was stored at 4 °C in H_2O under 4 drops of toluene (to prevent microbial growth).

Protein Determinations. Protein concentrations were determined by UV–biuret assay at 310 nm (Zamenhof, 1957) or by assuming an extinction at 280 nm of 1.0 at a protein concentration of 1 mg/mL when exact protein concentrations were not required.

Enzyme Assays. GAR transformylase was assayed by a spectrophotometric assay with 10-formyl-5,8-dideazafolate as the formyl donor. Solutions of 0.234 mM GAR, 0.010 mM 10-formyl-5,8-dideazafolate in 50 mM potassium phosphate, and 25% glycerol, pH 7.5, were incubated 10 min at 37 °C. Assays were started with enzyme, and the reaction was monitored by following the increase in absorbance at 295 nm. GAR transformylase activity was quantitated with $\Delta\epsilon_{295}$ = 18 900 M^{-1} cm^{-1} (Smith et al., 1981b).

The initial velocity studies on GAR transformylase with (6*R*)-10-CHO-H_4folate as the formyl donor were performed by using the spectrophotometric assay developed by Smith et al. (1981b). Assays were 0.02–2.0 mM in GAR and 2.0–85 μM in (6*R*)-10-CHO-H_4folate.

Initial velocity studies on GAR transformylase with 10-formyl-5,8-dideazafolate were done in preincubated solutions of GAR and 10-formyl-5,8-dideazafolate in 50 mM potassium phosphate, pH 6.8, and started with enzyme. Activity was quantitated as described above. Assays were 0.100–2.50 mM in GAR and 1.0–10.0 μM in 10-formyl-5,8-dideazafolate.

Sucrose Density Ultracentrifugation. This technique was performed following the method of Martin & Ames (1961) in 5–20% sucrose gradients in 50 mM Tris-HCl, 25% glycerol, and 10 mM 2-ME, pH 7.5 (33-mL total volume). Gradients

were poured at room temperature and allowed to equilibrate at 4 °C overnight. Centrifugation was performed on 0.35–0.8 mL of enzyme (0.05–0.1 mg) at 4 °C in a Beckman Model L5-50 preparative ultracentrifuge equipped with an SW-27 swinging-bucket rotor. Centrifugation at 25 000 rpm was carried out for 48 h. The molecular weight of GAR transformylase was determined by using yeast alcohol dehydrogenase and pig heart malate dehydrogenase as internal standards.

Enzyme Purification. All purification steps were carried out at 4 °C.

Extraction and Homogenization. About 60 g of liver tissue was obtained from 2-week-old chicks grown on a high-protein diet (Evans & Scholtz, 1971) to enhance GAR transformylase production (W. T. Mueller, unpublished results). This tissue was sliced and homogenized in batches of 30 g in 25 mL of 0.25 M sucrose, 10 mM potassium phosphate, and 1 mM EDTA, pH 7.5, containing 25 mg/L type II-O ovomucoid trypsin inhibitor, 25 mg/L α-1-antitrypsin, 15 mg/L pepstatin A, and 2.5 mL/L aprotinin. After the homogenate was adjusted to pH 7.5 with 0.1 M NH_4OH, it was spun at 30 000 rpm (100000g) for 90 min. The supernatnat was filtered through glass wool.

10-Formyl-5,8-dideazafolate–Sepharose Chromatography. About 50–70 mL of this filtered superantant was stirred with portions of dry 10-formyl-5,8-dideazafolate–Sepharose that had been preequilibrated with 10 mM potassium phosphate and 25% glycerol, pH 6.8. To determine how much GAR transformylase was bound to the resin, 5-mL samples were withdrawn and centrifuged, and the supernatants were assayed for enzyme activity. More dry resin was added as needed to bind more activity. In this way it was determined that 1 mL of dry resin bound 2.86 μmol/min enzyme activity. After this batch loading, the resin slurry was poured into a column containing about 7 mL of fresh column material packed in homogenization buffer (see above). The 3.5 × 15 cm column was connected to a reservoir of 10 mM potassium phosphate–25% glycerol, pH 7.1. Washing with this buffer continued until the red homogenate color was removed.

The resin was then washed with 10 mM potassium phosphate, 1.0 M KCl, and 25% glycerol, pH 7.1, until the OD_{280} of the eluant was less than 0.1. Finally, GAR transformylase was eluted with 30.0 mM potassium phosphate, 1.0 M urea, and 25% glycerol, pH 7.1.

Stoichiometry. The stoichiometry of GAR transformylase (10-CHO-H_4folate to FGAR) was determined by measuring first the (6R)-10-CHO-H_4folate consumed over 14 min and then the FGAR produced over the same time interval. For the (6R)-10-CHO-H_4folate determination, a 0.4-mL degassed solution 0.5 mM in GAR and 0.030 mM in (6R)-10-CHO-H_4 folate was incubated at 25 °C for 2 min under nitrogen. After any basal rate was recorded, the reaction was started with 0.008 mL of GAR transformylase solution. The change in absorbance at 312 nm was measured, and 10-CHO-H_4folate utilized was quantitated by using $\Delta\epsilon_{312}$ = 12 000 M^{-1} cm^{-1} as the difference in molar absorptivity between H_4folate and 10-CHO-H_4folate (Smith et al., 1981b). For the determination of FGAR production, (6R)-10-^{14}CHO-H_4folate was employed as above. After 14 min of reaction, the mixture was quenched with 0.071 mL of 0.5 N NaOH and then loaded onto a 0.55 × 12.5 cm column of QAE-Sephadex A-25 that had been equilibrated with 0.01 M $NaHCO_3$, pH 9.9. [^{14}C]FGAR was eluted with 0.02 M $NaHCO_3$, pH 9.9. The collected fractions were counted in a Beckman liquid scintillation counter, and FGAR was quantitated by using the known specific radioactivity in milliCuries per formyl group.

Antibody Production. Denatured GAR transformylase and trifunctional protein for use as antigens were further purified by electrophoresis of 0.5–1.0 mg of partially purified protein on a 12 × 13 × 0.3 cm slab gel (Laemmli, 1970). After the gel was stained for 5–10 min (Weber et al., 1972), the correct protein band could be identified, and a strip containing it was cut out of the gel. The protein was extracted by agitation in 0.2% SDS overnight and then concentrated by lyophilization. Antisera for these two proteins and native AICAR transformylase were raised in two rabbits each with Freund's complete adjuvant according to Crowle (1973).

^{14}C Labeling of Proteins. GAR transformylase and trifunctional protein were labeled with [^{14}C]formaldehyde by the procedure of Dottavio-Martin & Ravel (1978). Dialysis was replaced by the centrifuge desalting technique of White (1979).

Immunoprecipitation of GAR Transformylase. A protein preparation containing GAR transformylase was mixed with a sample of this preparation labeled with ^{14}C (as described under Methods) and immunoprecipitated with 4 μL of antisera followed by 400 μL of 10% IgGsorb as described by Kessler (1976) with modifications (J. M. Taylor, personal communication). Antigens and antisera were incubated 2–3 h at 4 °C in an immunoprecipitation buffer containing 1% NP-40, 0.15 M NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. After addition of the IgGsorb suspension, the protein A bound complex was centrifuged. Half of the supernatant fraction was assayed for GAR transformylase activity, and the other half was saved for electrophoresis. The protein A bound complex pellet was washed twice with 300 μL of immunoprecipitation buffer. Pellet-bound antigen was resolubilized by suspension in 50 μL of 0.125 M Tris-HCl, 20% glycerol (v/v), 6% SDS (w/v), and 10% 2-ME (v/v), pH 6.8, followed by heating to 95–100 °C for 5 min.

Solubilized antigen and supernatant fractions from above were electrophoresed on an 8.75% slab gel (Laemmli, 1970). Radioactive protein bands were detected by fluorography according to Ricca et al. (1981) with Kodak XAR-5 film.

Antibody Probing of Liver Homogenates by Western Blotting. (*A*) *Trichloroacetic Acid Homogenizations.* Homogenizations in TCA were done in a Brinkmann Polytron homogenizer. A 1-g sample of fresh liver was mixed with 10 mL of 10% TCA and homogenized. Homogenates were centrifuged at 12000g for 10 min at 4 °C. Pellets were taken and prepared for electrophoresis as follows. Precipitates were resuspended in 130 mM Tris-HCl, 10 mM sodium phosphate, 50 mM DTT, 1% (w/v) SDS, 10% (v/v) glycerol, and 0.004% (w/v) bromphenol blue, pH 9.2. The suspension was repeatedly vortexed, sonicated, and heated in a boiling water bath to facilitate solubilization. The pH was adjusted with Tris base (solid) when necessary. After this treatment, samples were diluted 12-fold into the above buffer (minus the Tris) and incubated at 100 °C for 5 min. Electrophoresis was done on 7.5- and 15-μL samples.

(*B*) *Normal Homogenizations.* These homogenizations were performed as described under Enzyme Purification by homgenizing 1 g of tissue in 10 mL of homogenization buffer. After centrifugation and filtration, samples were diluted 5-fold with water, and a one-fifth volume of 50 mM sodium phosphate, 250 mM DTT, 5% (w/v) SDS, 50% (v/v) glycerol, and 0.02% bromphenol blue, pH 70, was added. Homogenates were incubated for 5 min at 100 °C. Electrophoresis was done on 15- and 30-μL samples.

(*C*) *Electrophoresis.* SDS–polyacrylamide gel electrophoresis for the Western blotting technique was performed with

the gel system of Neville (1971) employing a 8.75% slab. Electrophoresis was done for 4.5 h at 125 V. After electrophoresis, the gel was washed 3 times with 300 mL of 15 mM sodium phosphate, pH 6.5, for 5 min to partially displace the Tris-borate buffer.

(*D*) *Electrophoretic Transfer of Proteins from Polyacrylamide to Paper* (*Western Blot*). This technique was done by the method of Reiser & Stark (1983) with a transfer apparatus prepared by John Farley (32 Oak St., Charleston, MA 02129). The apparatus is similar to the Trans-Blot Cell of Bio-Rad. One piece of APT paper was diazotized in 100 mL of ice-cold 1.2 N HCl containing 27 mg of $NaNO_2$ for 20 min. The paper was then washed with ice-cold water and ice-cold 15 mM sodium phosphate, pH 6.5.

To prepare for transfer, a 15 mM sodium phosphate saturated piece of Whatman 3MM paper was placed on the cathode pad of the transfer apparatus, and the polyacrylamide gel was placed on top of that. The diazotized APT paper was carefully placed on top of the gel, and it was covered by three 15 mM phosphate-saturated 3MM paper pieces. After air bubbles were quickly removed by rolling a glass rod over the top papers, the anode pad was put on top, and the entire assembly was inserted into the transfer chamber containing 4 L of chilled 15 mM sodium phosphate, pH 6.5. Transfer was accomplished at 20 °C by electrophoresis for 1 h at 2-A current.

(*E*) *Treatment of the Blot and Antibody Probing.* After transfer, the blotted paper was deactivated by immersion in 0.1 M Tris-HCl, 0.25% (w/v) gelatin, pH 9.0, overnight. The deactivated blot was immersed in 250 mL of 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.25% (w/v) gelatin, and 0.05% (v/v) NP-40, pH 7.4 (referred to as antibody buffer), for 10 min at 25 °C. This process was repeated twice, and the paper was sealed in a heat-sealable freezer bag with 5 mL of antibody buffer containing 120 μL of anti-GAR that had been filtered through a 0.45-μm Millipore GV filter. After being sealed, the bag was rotated end over end at 25 °C for 1 h. The anti-GAR-treated paper was washed for 4 h in antibody buffer with six buffer changes.

The blot was sealed in a bag with 5 mL of antibody buffer containing 1 μCi of ^{125}I-labeled protein A and rotated 1 h at 25 °C. After removal from the bag, the paper was washed 4 times with 250 mL of 0.65 M NaCl, 5 mM EDTA, 50 mM tris-HCl, and 0.4% (w/v) *N*-laurylsarcosine, pH 7.4, over 1 h. The moist paper was covered with plastic wrap and autoradiographed at −70 °C on Kodak XAR-5 film with a Du Pont Cronex Lightning-Plus intensifier screen.

(*F*) *Limited Proteolysis of GAR Transformylase.* (*1*) *With Liver Extract.* Liver extract prepared by homogenization of 5 g of chicken liver in 30.0 mL of 10 mM Tris-HCl, pH 7.5, in a Waring blender followed by centrifugation at 100000*g* for 90 min was mixed with 0.050 mL of [^{14}C]GAR transformylase solution (0.023 mg). At specified times, samples were drawn and frozen in liquid nitrogen. After storage, all samples were thawed and boiled in 0.125 M Tris-HCl, 20% glycerol, 6% SDS, and 10% 2-ME, pH 6.8, for 5 min. The denatured proteins were electrophoresed on an 8.75% polyacrylamide–SDS gel. Radioactive protein bands were detected by fluorography (Ricca et al., 1981) on Kodak XAR-5 film.

(*2*) *With Commercial Protease Preparations.* The proteases used were chosen for their endopeptidase activity and their appreciable activity at pH 7.5. Protease solutions were made up in 7.5 mM potassium phosphate, pH 7.5, as stocks immediately before use. A 0.104-mL solution of GAR transformylase (0.062 mg) was mixed with 0.146 mL of protease solution to give a 500-fold molar excess of GAR transformylase. At specified times, 0.020-mL samples were withdrawn and denatured as described above.

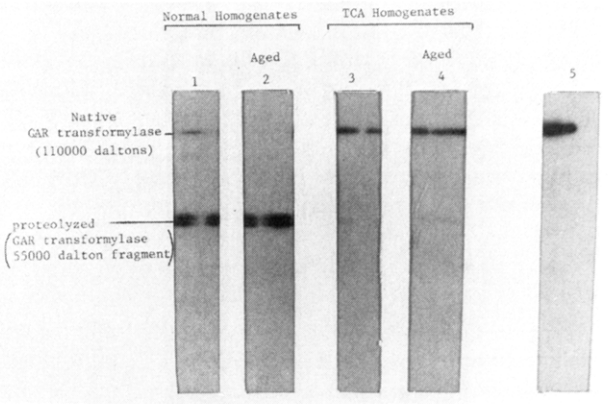

FIGURE 1: Probe of a Western blot of various liver homogenates with anti-GAR as described under Methods. Lanes 1 and 2 are homogenates prepared by the normal homogenization, as for an enzyme purification. Lanes 3 and 4 are homogenates prepared in trichloroacetic acid. Samples marked aged were incubated 2 h at 0 °C before homogenization. Lane 5 is purified GAR transformylase.

Trypsin, chymotrypsin, and subtilisin BPN′ were the proteases used. In the case of chymotrypsin, 0.020-mL samples were assayed for GAR transformylase activity in the given times. The denatured proteins were electrophoresed on 8.75% SDS–polyacrylamide slab gels (Laemmli, 1970).

Results

Identification of GAR Transformylase in Liver Homogenates by Antibody Probe. Two types of samples were homogenized in both TCA and normal homogenization buffer: (1) liver that was homogenized immediately after excision and (2) liver that was incubated 2 h at 0 °C before homogenization (described as "aged"). After homogenization, these samples were denatured and electrophoresed. The samples were then probed with antibodies by the Western blot procedure as described above. These results are presented in Figure 1.

The TCA homogenates should reflect accurately the state of the protein in vivo, since the use of TCA in homogenization should reduce any proteolytic artifacts. Probes of these homogenates show that the transformylase is a protein of about 110000 daltons almost exclusively (lanes 3 and 4). This compares well with a more purified GAR transformylase preparation in lane 5. In contrast, the protein obtained by our normal homogenization procedure is primarily in the 50000–55000-dalton range (lanes 1 and 2). Furthermore, the aged sample (lane 2), which was incubated 2 h before homogenization, has no 110000-dalton protein antigenic to anti-GAR. Since the TCA-homogenized liver has primarily 110000-dalton antigen, this suggests that GAR transformylase is proteolyzed to give smaller peptides as a result of proteases released upon homogenization. All the lanes should have had the same amount of total GAR transformylase. The samples from the TCA homogenates are consistently lower in GAR transformylase, probably as a result of incomplete resolubilization of proteins.

Enzyme Purification. This purification procedure supplants our previous purification for GAR transformylase (Caperelli et al., 1980). While we were able to purify both GAR transformylase and the trifunctional enzyme with the earlier procedure, the new method is much faster and technically simpler. The saving in time is about 1 week. Typically, it is possible to obtain nearly pure GAR transformylase in 8 h. The

Table I: Purification of GAR Transformylase

step	vol (mL)	total protein (mg)	act. (μmol/min)	sp act. (μmol min^{-1} mg^{-1})	x-fold purification (% yield)
homogenate supernatant	75	8.1	286	0.035	1 (100)
10-formyl-5,8-dideazafolate–Sepharose	113	0.0085	126	14.8	424 (44)

Table II: Steady-State Kinetic Parameters of GAR Transformylase

cofactor	temp (°C)	V_{max} (μmol mg^{-1} min^{-1})	K_{GAR} (μM)	$K_{cofactor}$ (μM)
(6*R*)-10-CHO-H_4folate	37	25.1 ± 2.3	244 ± 53	10.0 ± 3.0
10-formyl-5,8-dideazafolate	37	7.8	359 ± 76	0.75

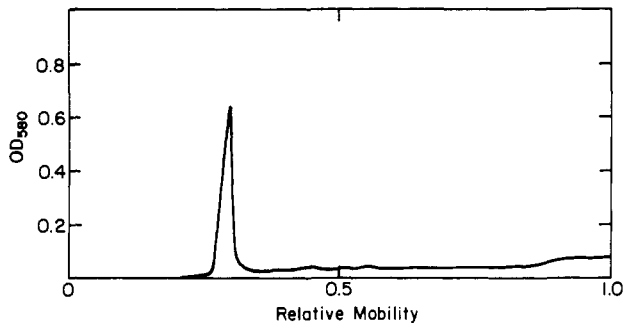

FIGURE 2: Scanning densitometer trace of an SDS–polyacrylamide electrophoresis gel of GAR transformylase purified as described under Methods.

purification results are summarized in Table I. Figure 2 shows a scanning densitometer trace of the purified enzyme.

Two other proteins of interest, namely, trifunctional protein and AICAR transformylase (Mueller & Benkovic, 1981), do not appear to bind to the affinity column, as they elute in the 10 mM potassium phosphate and 1 M KCl wash steps. AICAR transformylase may be further purified by chromatography of fractions from the 10 mM potassium phosphate wash on Matrex Gel Red A (Amicon).[2] No further attempts to purify the trifunctional protein were made.

Molecular Weight Determination. GAR transformylase activity was assigned to a protein of subunit molecular weight 110000 on the basis of SDS–polyacrylamide gel results. This is in contrast with the subunit molecular weight of GAR transformylase of 55000 reported previously (Caperelli et al., 1980). In order to investigate the subunit structure of GAR transformylase, sucrose density ultracentrifugation was undertaken according to Martin & Ames (1961). The overall molecular weight of GAR transformylase could be estimated at 140000, from the standards as shown in Figure 3. Active enzyme centrifugation experiments (Kemper & Everse, 1973) indicate that the active form of the enzyme is 110000–120000 daltons. Thus, a one-subunit structure is indicated. An average of all values obtained so far yields and overall molecular weight of approximately 117000 (data not shown).

Steady-State Kinetic Parameters. The steady-state kinetic parameters for GAR transformylase at 37 °C appear in Table II. Each set of parameters was determined from nonlinear regression of one initial velocity pattern. Double-reciprocal plots were nonparallel; thus the data were fit to a sequential kinetics scheme by employing the FORTRAN program of Cleland (1967).

Stoichiometry. The stoichiometry of GAR transformylase [(6*R*)-10-CHO-H_4folate to FGAR] was determined by quantitating both the (6*R*)-10-CHO-H_4folate used and the FGAR produced in the reaction. The ratio of these two quantities was 1:1.

[2] M. Young, unpublished results.

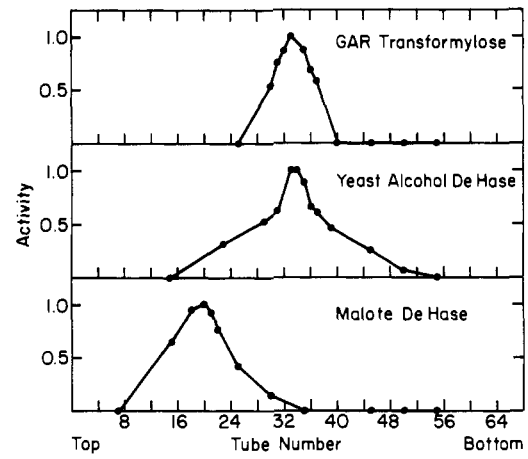

FIGURE 3: Sucrose density ultracentrifugation of GAR transformylase compared with yeast alcohol dehydrogenase and malate dehydrogenase. Centrifugation was done at 25000 rpm for 48 h at 0–4 °C in 5–20% sucrose gradients. After centrifugation, the tube contents were withdrawn, fractionated into 0.5-mL fractions, and assayed for each enzyme activity. Calculations were done according to Martin & Ames (1963) by assuming a spherical shape for each protein. The designations top and bottom refer to position in the ultracentrifuge tube. The experiment was performed in duplicate, and results were identical between the two tubes.

Table III: Immunoprecipitation of GAR Transformylase with Anti-GAR and Preimmune Serum

anti-serum	% activity in supernatant
anti-GAR	<10
anti-M	>90
preimmune	>99

Immunoprecipitation of GAR Transformylase Activity. To assess the specificity of our antibody for GAR transformylase, we attempted the immunoprecipitation of GAR transformylase by anti-GAR from a preparation containing a number of proteins. Immunoprecipitation by preimmune serum (serum prepared from nonimmunized rabbits) or antiserum raised to an unrelated protein in the mixture (referred to as M) served as controls. A sample of the crude preparation was immunoprecipitated with anti-GAR, preimmune serum, and anti-M. After centrifuging the bound IgG–protein–IgGsorb pellet was centrifuged and the supernatant fraction was drawn off, half the supernatant was assayed for GAR transformylase activity. Table III summarizes the results. Little or no activity is precipitated by preimmune or anti-M sera, but more than 90% of the activity is precipitated by the anti-GAR. The precipitated proteins were released from the pellet by boiling in SDS–2-ME solution and electrophoresed on an SDS–polyacrylamide slab gel. Radioactive proteins were discerned by fluorography as described under Methods. Although a large amount of nonspecific binding was observed, the precipitation of the GAR transformylase band at 117000 daltons correlated with the reduction in supernatant GAR transformylase activity (data not shown).

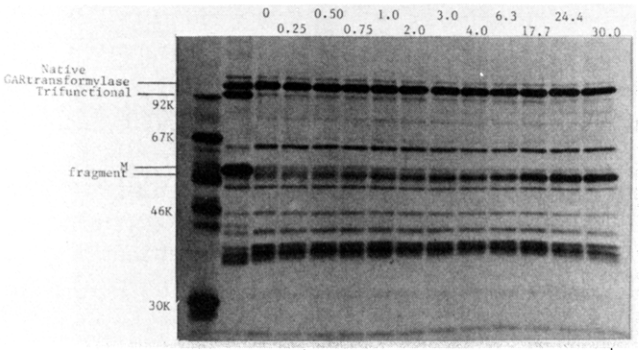

FIGURE 4: Proteolysis of GAR transformylase with liver homogenate. After [^{14}C]GAR transformylase was mixed with liver homogenate as described under Methods, 20-μL samples were drawn at the indicated times (in hours) and quenched by freezing under liquid nitrogen. Other bands arise from proteins present in the partially purified GAR transformylase preparation used in ^{14}C labeling. The second lane from the left contains the native GAR transformylase, trifunctional protein, and a contaminating protein, M. The leftmost lane contains the molecular weight markers referred to at left.

Limited Proteolysis of GAR Transformylase. (*1*) *Liver Extract Proteolysis.* The results of incubation of [^{14}C]GAR transformylase with chicken liver extract are presented in Figure 4. It is apparent that a protein fragment of 55 000 daltons increases with time, and that this fragment originates from GAR transformylase, or from a protein too large to enter the gel. Lower percentage (5%) polyacrylamide gels do not reveal such large proteins, and chymotryptic proteolysis of similar but purer preparations does not produce an anomalous amount of cleavage product, indicating the presence of a large protein is unlikely. Thus some liver protease is capable of degrading GAR transformylase as was indicated by the Western blot results.

(*2*) *Purified Proteases.* Results of proteolysis of GAR transformylase with subtilisin BPN′, chymotrypsin, and trypsin are presented in Figure 5. All three digests are qualitatively similar, featuring the formation of fragments in the 50 000–55 000-dalton range. Subtilisin is the most unique, forming predominantly a 30 000-dalton fragment so that 50 000–55 000-dalton fragments are less than 50% of total fragments. Chymotrypsin produces two fragments each of which has its comigrating analogue in the subtilisin pattern. Trypsin digestion yields three bands in the 50 000–55 000-dalton range, two of which are analogous to fragments in the other digestion patterns. Taken together, these results are consistent with a model of cleavage at one site forming peptides of equal size followed by partial digestion of the formed fragments.

Table IV: Activity of GAR Transformylase during Proteolysis

time after protease addition	activity[a]	extent of proteolysis (%)[b]
0 min	0.0266	0
16 min	0.0266	10
30 min	0.0305	20
47 min	0.0292	40
64 min	0.0323	60
120 min	0.0288	85
5 h	0.0276	100

[a] Activity is expressed as ΔOD_{295}/min at 37 °C. [b] Extent of proteolysis is a visual estimate from a polyacrylamide gel. See Results.

A comparison of the two systems shows (1) the liver protease produces only one visible proteolysis fragment while the non-liver proteases produce a spectrum of protein bands and (2) in all cases a fragment at 55 000 daltons is seen, which may indicate a protease-labile domain within GAR transformylase.

The fact that the chymotrypsin digestion of ^{14}C-labeled GAR transformylase generates a fragment pattern identical with the unlabeled fragment pattern[2] suggests that the liver protease digestion pattern is a reasonably accurate accounting of fragments and that the cleavage points for the two proteases on GAR transformylase are somewhat different.

(*3*) *Activity of Chymotrypsin Digests.* In a separate experiment the remaining GAR transformylase activity and the extent of proteolysis by chymotrypsin were monitored as a function of time (Table IV). The proteolysis time course is given as percent fragments and is a visual estimate from the gel. The fragmentation pattern was identical with that in Figure 4. The results clearly show that full activity is maintained even when proteolysis is 100% complete (i.e., no protein

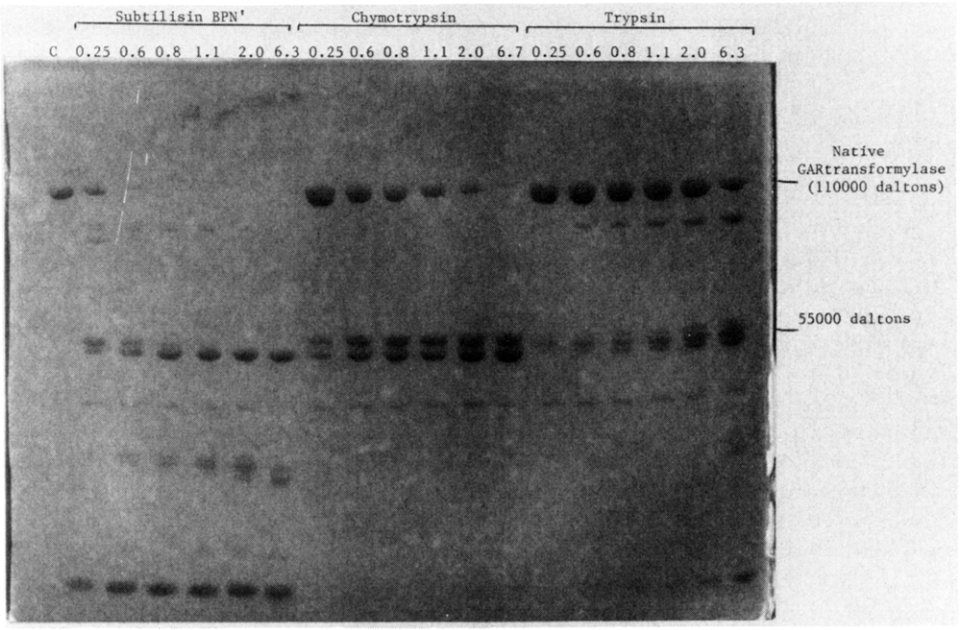

FIGURE 5: Proteolysis of GAR transformylase with commercially available proteases. Samples of proteases were each mixed with GAR transformylase, and 20-μL samples were withdrawn and denatured at the times shown in hours. Samples were electrophoresed on an 8.75% polyacrylamide gel, and the gel was stained according to the method of Weber et al. (1972). Lane C contains purified GAR transformylase with no added protease.

band at 117 000 daltons is observed).

Discussion

The Western blot technique allows us to directly probe any given preparation for the presence of GAR transformylase and its degradation products. Using this method, we have been able to detect proteolysis of this protein during our homogenization procedure to a 55 000-dalton degradation product. Evidence that the 117 000-dalton protein purified by us is the native GAR transformylase can be found in the probes of Western blots of TCA-homogenized livers. That the TCA prevents artifactual proteolysis can best be seen in a comparison of the aged homogenates (Figure 1). The aged normal homogenates contain no native GAR transformylase, while the aged TCA homogenates do contain the native protein and do not contain degradation products. This shows that any degradation in the normal homogenates must happen after homogenization; therefore, TCA does prevent proteolysis after homogenization. Furthermore, the observation that anti-GAR precipitates more than 95% of the GAR transformylase activity from the liver homogenate is in accord with a single protein being the source of this activity.

We have performed two additional antibody probes of this blot, one using antiserum to trifunctional protein and one using antiserum to AICAR transformylase (data not shown). The anti-AICAR probe shows that the largest peptide antigenic to anti-AICAR is the 67 000-dalton peptide previously identified and purified by Mueller & Benkovic (1981) as one subunit of AICAR transformylase. The anti-TP probe reveals the largest anti-TP antigen present to be a peptide of 95 000 daltons. This is in agreement with the subunit molecular weight of 90 000–97 000 determined by Smith et al. (1980). The anti-AICAR probe displays a pattern of antigenic bands below 67 000 daltons, possibly indicating some proteolysis of this enzyme. These results confirm the native molecular weights of AICAR transformylase and trifunctional protein and serve to indicate a property of GAR transformylase rendering it particularly susceptible to proteolytic cleavage.

The purification of native GAR transformylase has been accomplished by two methods in our laboratory. Initially the protein was purified by starting with a protein preparation obtained after the AICAR–Sepharose chromatography step in the procedure of Smith et al. (1980). A column of Cibacron Blue F3GA–agarose (Sigma) was employed to obtain a preparation of GAR transformylase that was 85–95% pure by the criterion of SDS–polyacrylamide gel electrophoresis. The binding of GAR transformylase to Cibacron Blue–agarose is somewhat surprising, but the binding of folate-requiring enzymes to Cibacron Blue has precedence in the work of Wilson (1976), Subramanian & Kaufman (1980), and Stellwagen et al. (1975). On the other hand, the binding of GAR transformylase to the column of 10-formyl-5,8-dideazafolate–Sepharose was anticipated since the column ligand is a substrate for the enzyme (Table II). The apparent specificity of the column for GAR transformylase, manifested by a large purification in a single step, was gratifying. This single-step purification is completely equivalent to our previously used procedure, which required five steps. The increased yield allows us to obtain much larger amounts of protein in a shorter time with less tissue.

Caperelli et al. (1978) assigned GAR transformylase activity to a dimer of 55 000-dalton subunits because of their purification of such a dimer to homogeneity. Their ultracentrifugation results indicated an overall molecular weight of 110 000. Due to the facts that proteolyzed GAR transformylase remains fully active and that a number of proteases produce 55 000-dalton fragments (among them a chicken liver protease), it appears that the protein isolated by Caperelli et al. (1978) is the 55 000-dalton fragment. Overall molecular weights of 110 000 (Caperelli et al., 1978) could result from association of cleavage fragments in nondenaturing buffers similar to the association of fragments of the cleaved chain of ribonuclease A and chymotrypsinogen to form the active enzymes ribonuclease S and α-chymotrypsin (Dickerson & Geis, 1969; Miller et al., 1971).

The purification and identification of the active form of GAR transformylase as well as the verification of the native species of AICAR transformylase and the trifunctional protein now allow us to further pursue questions of mechanism and possible interactions between the proteins. The one-step purification of GAR transformylase greatly increases the availability of pure protein and also holds the promise of being applicable to other species.

Acknowledgments

We thank P. A. Benkovic for performing the stoichiometry experiments, Dr. S. C. Daubner for her technical assistance, advice, and discussion, and Dr. J. T. Chen for his technical assistance.

Registry No. 10-CHO-H_4folate, 74644-66-9; GAR transformylase, 9032-02-4; 10-formyl-5,8-dideazafolate, 61038-31-1; 5,8-dideazafolate, 5854-11-5; 2-amino-6-(bromomethyl)-4-hydroxyquinazoline, 58677-08-0; diethyl (4-aminobenzoyl)-L-glutamate, 90791-08-5.

References

Acharya, S. P., & Hynes, J. B. (1975) *J. Heterocycl. Chem. 12*, 1283.
Blakley, R. L. (1960) *Nature (London) 188*, 231.
Caperelli, C. A., Chettur, G., Lin, L. Y., & Benkovic, S. J. (1978) *Biochem. Biophys. Res. Commun. 82*, 403.
Caperelli, C. A., Benkovic, P. A., Chettur, G., & Benkovic, S. J. (1980) *J. Biol. Chem. 255*, 1885.
Chettur, G., & Benkovic, S. J. (1977) *Carbohydr. Res. 56*, 75.
Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1.
Crowle, A. J. (1973) *Immunodiffusion*, Academic Press, New York.
Cuatrecasas, P., & Anfinsen, C. B. (1971) *Methods Enzymol. 22*, 345.
Dickerson, R. E., & Geis, I. (1969) *The Structure and Action of Proteins*, p 80, Harper and Row, New York.
Dottavio-Martin, D., & Ravel, J. M. (1978) *Anal. Biochem. 87*, 562.
Evans, R. M., & Scholz, R. W. (1971) *J. Nutr. 101*, 1127.
Kemper, D. L., & Everse, J. (1973) *Methods Enzymol. 27*, 67.
Kessler, S. W. (1976) *J. Immunol. 117*, 1482.
Laemmli, U. (1970) *Nature (London) 227*, 680.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.
Martinelli, J. E., & Chaykovksy, M. (1980) *Prep. Biochem. 10*, 161.
Mathews, C. K., & Huennekens, F. M. (1960) *J. Biol. Chem. 235*, 3304.
Miller, D. D., Horbell, T. A., & Teller, D. C. (1971) *Biochemistry 10*, 4641.
Mueller, W. T., & Benkovic, S. J. (1981) *Biochemistry 20*, 337.
Neville, D. M. (1971) *J. Biol. Chem. 246*, 6328.
Reiser, J., & Stark, G. R. (1983) *Methods Enzymol. 96*, 205.
Renart, J., Reiser, J., & Stark, G. (1979) *Proc. Natl. Acad.*

Sci. U.S.A. 76, 3116.
Ricca, G. A., Hamilton, R. W., McLean, J. W., Conn, A., Kalinyak, J. E., & Taylor, J. M. (1981) *J. Biol. Chem. 256*, 10362.
Rode, M., Scanlon, K. J., & Bertino, J. R. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) Vol. 4, pp 489–494, Elsevier/North-Holland, New York.
Rowe, P. B. (1968) *Anal. Biochem. 22*, 166.
Seed, B. (1982) *Nucleic Acids Res. 10*, 1799.
Smith, G. K., Mueller, W. T., Wasserman, G. F., Taylor, W. D., & Benkovic, S. J. (1980) *Biochemistry 19*, 4313.
Smith, G. K., Mueller, W. T., Benkovic, P. A., & Benkovic, S. J. (1981a) *Biochemistry 20*, 1241.
Smith, G. K., Benkovic, P. A., & Benkovic, S. J. (1981b) *Biochemistry 20*, 4034.
Subramanian, S., & Kaufman, B. T. (1980) *J. Biol. Chem. 255*, 10587.
Thompson, S. T., Cass, K. H., & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 669.
Warren, L., & Buchanan, J. M. (1957) *J. Biol. Chem. 229*, 613.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods. Enzymol. 26*, 3.
White, J. C. (1979) *J. Biol. Chem. 254*, 10889.
Wilson, J. E. (1976) *Biochem. Biophys. Res. Commun. 72*, 816.
Zamanhof, S. (1957) *Methods Enzymol. 3*, 696.

Rod Outer Segment Phosphodiesterase Binding and Activation in Reconstituted Membranes†

Patricia N. Tyminski and David F. O'Brien*

ABSTRACT: Light exposure of rhodopsin in rod outer segment (ROS) membranes activates several cyclic GMP phosphodiesterase (PDE) molecules via a GTP-binding protein (G protein). Both PDE and G protein are surface-associated (peripheral) enzymes, which may be extracted from ROS by hypotonic media, individually purified, and recombined in isotonic media with purified rhodopsin–phospholipid vesicles to yield membranes of low dark and high light phosphodiesterase activity. In isotonic media, the PDE strongly associates with phospholipid membranes as well as with ROS and rhodopsin–phospholipid membranes. Because only membrane-associated PDE is readily light activated, the PDE activity saturates when the available binding sites are occupied. At a constant G-protein concentration, the PDE activity observed at saturation is 4 times greater for unilamellar rhodopsin–phospholipid vesicles with a lipid to rhodopsin ratio of 460 than for those with a ratio of 120. Thus, PDE association with membrane in isotonic media is dependent on the phospholipid content rather than the rhodopsin content. Several G proteins per PDE are necessary to maximize the PDE activity of reconstituted membranes; therefore, a weak association between activated G protein and PDE is indicated. Both peripheral enzymes readily transfer between membrane surfaces. Rhodopsin–phospholipid vesicles devoid of enzyme activity were exposed to a light flash and then mixed in the dark in isotonic media with unilluminated ROS membranes which contained PDE and G protein. PDE activity was observed within 2 s after mixing. Subsequent separation and evaluation of the denser ROS membranes and the less dense vesicles demonstrated that both PDE and G protein were associated with the vesicles as well as the ROS membranes. This peripheral protein transfer is at least 10^3 times faster than previously identified lipid exchange between vesicles or the transfer of integral protein between membranes and vesicles. The transfer may be fast enough to be physiologically relevant in this membrane system, and similar transfer processes are likely in experiments with other peripheral proteins.

Light absorption by the 11-*cis*-retinal of the transmembrane rhodopsin (Rh)[1] produces spectral and structural changes in the protein which result in the activation of several photoreceptor outer segment enzymes (O'Brien, 1982; Pober & Bitensky, 1979). In one activation sequence, a photoproduced intermediate of rhodopsin (Rh*) interacts with the membrane surface associated G protein to facilitate the exchange of GTP for GDP (Fung & Stryer, 1980). This G-protein complex (G_{GTP}) in turn activates a second membrane surface associated enzyme, a cGMP phosphodiesterase (PDE) (Fung et al., 1981), which catalyzes the hydrolysis of cGMP with a turnover of 10^3 s^{-1} (Yee & Liebman, 1978). The two-step amplification (a single Rh* may catalyze the binding of GTP to $\sim 10^2$ G proteins, each of which may activate a PDE) results in the hydrolysis (in vitro) of 10^5 cGMP per Rh* per s (Liebman & Pugh, 1979; Woodruff & Bownds, 1979).

A current hypothesis for the activation sequence is given by the following equations, where G_{GDP} represent G protein with bound GDP and G_{GTP}* symbolizes G protein with bound GTP, with the asterisk indicating an activated species. More information is necessary about this sequence of reactions and the reactants before a kinetic theory can be formulated for the activation and inactivation of Rh, G, and PDE. It is known that rhodopsin is a transmembrane protein (Fung & Hubbell,

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[1] Abbreviations: ROS, rod outer segment(s); Rh, rhodopsin; PDE, phosphodiesterase; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; cGMP, guanosine cyclic 3′,5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; G protein, GTP-binding protein; SDS, sodium dodecyl sulfate; GTPase, guanosinetriphosphatase; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

0006-2960/84/0423-3986$01.50/0