A Multifunctional Protein Possessing Glycinamide Ribonucleotide Synthetase, Glycinamide Ribonucleotide Transformylase, and Aminoimidazole Ribonucleotide Synthetase Activities in de Novo Purine Biosynthesis†

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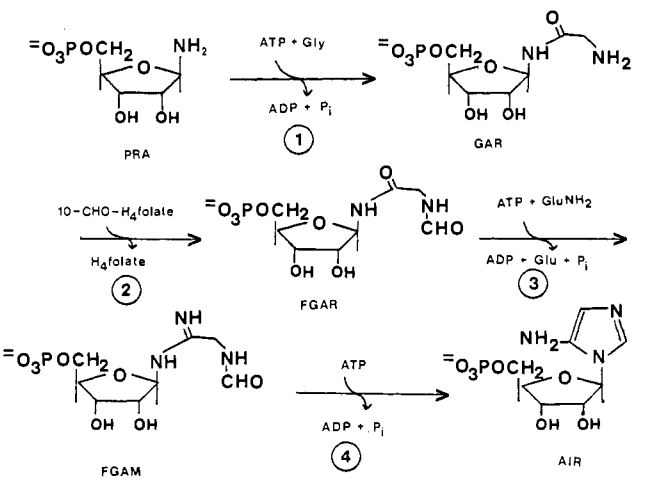
ABSTRACT: Three activities on the pathway of purine biosynthesis de novo in chicken liver, namely, glycinamide ribonucleotide synthetase, glycinamide ribonucleotide transformylase, and aminoimidazole ribonucleotide synthetase, have been found to reside on the same polypeptide chain. Three diverse purification schemes, utilizing three different affinity resins, give rise to the same protein since the final material has identical specific activities for all three enzymatic reactions and a molecular weight on sodium dodecyl sulfate gels of about 110000. A single antibody preparation precipitates all three activities and binds to the multifunctional protein obtained by two methods in Western blots. Partial chymotryptic digestion of the purified protein gives rise to two fragments, one possessing glycinamide ribonucleotide synthetase activity and the other containing glycinamide ribonucleotide transformylase activity.

Various lines of biochemical and genetic evidence suggested that three activities of purine de novo biosynthesis, GAR[1] synthetase, GAR transformylase, and AIR synthetase, could be properties of the same protein species. It was demonstrated by Henikoff et al. (1983) that GAR transformylase from *Drosophila* is encoded by 1 kb of a 4.76-kb mRNA so that the transformylase is the carboxyl-terminal domain of a much larger polypeptide. Complementation studies on the *ade*5 and *ade*7 mutants of yeast that lacked GAR synthetase and AIR synthetase activities suggested that these activities might reside on a single polypeptide or in an enzyme complex (Gross & Woods, 1971); copurification of GAR synthetase and AIR synthetase activities from *Schizosaccharomyces pombe* substantiated that hypothesis (Fluri et al., 1976). Other work by Henikoff et al.[2] showed that the same genetic locus encoding GAR transformylase encodes GAR synthetase and AIR synthetase in *Drosophila* and suggested that in this organism the three enzyme activities are carried on a single protein. Subsequent studies carried out in mammalian systems (Patterson et al., 1981, 1982) demonstrated that GAR synthetase and AIR synthetase activities could be coordinately altered by mutations in mammalian cells and assigned to the same human chromosome. The portion of the de novo purine biosynthetic pathway in which these activities occur is shown in Scheme I (Buchanan & Hartman, 1973). The results of this joint inquiry on a protein isolated from chicken liver by two different groups with four different protocols are the subject of this paper.

Scheme I: Steps of IMP Biosynthesis That Convert Phosphoribosylamine to Amidoimidazole Ribonucleotide[a]



[a] The enzymes involved are indicated by number: 1, GAR synthetase; 2, GAR transformylase; 3, FGAR amidotransferase; 4, AIR synthetase.

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[1] Abbreviations: GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; AIR, aminoimidazole ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide; NADH, nicotinamide adenine dinucleotide; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; kb, kilobase; GAR transformylase, glycinamide ribonucleotide formyltransferase; GAR synthetase, glycinamide ribonucleotide synthetase; AIR synthetase, aminoimidazole ribonucleotide synthetase; FGAR amidotransferase, formylglycinamide ribonucleotide amidotransferase; IMP, inosine monophosphate.

[2] S. Henikoff, D. Patterson, J. S. Sloan, J. Bleskan, R. Hards, and M. A. Keene, unpublished results.

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Experimental Procedures

Materials

SDS gel electrophoresis marker proteins bovine serum albumin, ribonuclease A, ATP, D-ribose 5′-phosphate, glycine, ATP–agarose (linked through N-6 of the adenine ring by a 6-carbon spacer), DEAE-Sephadex A-25, Sephadex G-200, pyruvate kinase (355 μmol min^{-1} mg^{-1}), lactate dehydrogenase (920 μmol min^{-1} mg^{-1}), phosphoenolpyruvate, NADH, and TPCK were obtained from Sigma Chemical Co. Hydroxylapatite was purchased from Bio-Rad Laboratories. [2-^{14}C]-Glycine was obtained from Amersham. Chymotrypsin was obtained from Worthington. ^{125}I-Labeled protein A was purchased from New England Nuclear. 10-Formyl-5,8-dideazafolate and 10-formyl-5,8-dideazafolate–Sepharose were prepared as previously described (Young et al., 1984). AIR was prepared by the procedure of Schrimsher and Stubbe.[3] GAR was synthesized by the procedure of Chettur & Benkovic (1977).

Synthesis of FGAM. FGAR (a 1:1 mixture of anomers determined by NMR spectroscopy) was synthesized by extensive modifications of the procedure of Chu & Henderson (1970). FGAR amidotransferase (specific activity 0.25 mol min^{-1} mg^{-1}) was purified from chicken liver according to the procedure of Schendel and Stubbe.[4] The reaction mixture for the synthesis of FGAM consisted of 2.5 mM ATP, 1.0 mM FGAR, 3.0 mM glutamine, 5.0 mM Tris-HCl, 60 mM KCl, and 2.0 units of FGAR amidotransferase in a volume of 60 mL. The pH of the reaction mixture was adjusted to 8.0 before the addition of FGAR and enzyme. The reaction mixture was incubated at 37 °C for 30 min and subjected to ultrafiltration using an Amicon PM-30 membrane for the removal of protein. The filtrate was diluted to 250 mL and applied to a column of DEAE-Sephadex A-25 (2.5 × 27 cm), which was then washed with 2 column volumes of water. FGAM was eluted with a 1.0-L linear gradient of triethylammonium bicarbonate (pH 8.0, 0–400 mM). The fractions (15 mL) that contained FGAM, detected by assay for total phosphate (Ames & Dubin, 1960), were pooled and evaporated to dryness in vacuo.

Methods

Protein Determinations. Protein concentrations were determined by the methods of Lowry et al. (1951) and Zamenhof (1957). The refractive index of solutions of the purified GAR transformylase was compared to standard curves of bovine serum albumin and ribonuclease A in order to determine the former protein concentration (OD_{279} = 1.13 for 1 mg/mL).

Polyacrylamide Gel Electrophoresis. SDS gel electrophoresis (7.5% or 8.75% acrylamide) was performed according to the procedure of Laemmli (1970) or Neville (1971) with molecular weight standards that included β-galactosidase, phosphorylase *b*, bovine serum albumin, egg albumin, and carbonic anhydrase.

Western Blots. Electrophoretic transfer of proteins from SDS gels to paper was performed as described by Young et al. (1984). Antibody used in probing the blots was prepared in rabbits after injections of native mouse L1210 cell GAR transformylase (Daubner & Benkovic, 1985).

Immunoprecipitation of Protein. A purified protein sample (5–20 μg) containing AIR synthetase, GAR synthetase, and GAR transformylase activities was immunoprecipitated with antisera (prepared to chicken GAR transformylase) according to the procedure of Kessler (1976) as modified by Young et al. (1984). After centrifugation to remove precipitate, the supernatant was assayed for the three activities.

Enzyme Assays. GAR synthetase was assayed by one of two methods. The enzyme was assayed in the forward direction in a reaction mixture (0.5 mL) containing 100 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 20 mM D-ribose 5′-phosphate, 290 mM NH_4Cl, 1.0 mM ATP, and 10 mM [^{14}C]glycine (1×10^5 cpm/μmol). The reaction was incubated at 37 °C and initiated by the addition of enzyme. At 2-min intervals, including a zero time point, 100 μL of the reaction mixture was quenched with 15 μL of 30% (w/v) trichloroacetic acid and absorbed onto Dowex 50 W-X8 (NH_4^+ form, pH 3.3). The GAR produced was eluted with 2.5 mL of 50 mM ammonium formate and quantitated by scintillation counting of 1 mL of the 2.6-mL effluent.

GAR synthetase in the absence of GAR transformylase was assayed as an ATPase by coupling to pyruvate kinase and lactate dehydrogenase. Samples of enzyme were added to 1-mL solutions containing 11 mM D-ribose 5′-phosphate, 32 mM NH_4Cl, 11 mM glycine, 0.725 mM ATP, 6 mM $MgCl_2$, 0.204 mM NADH, 0.37 mM phosphoenolpyruvate, 14.6 units of lactate dehydrogenase, 3.4 units of pyruvate kinase, and 80 mM Tris-HCl, pH 8.0 at 37 °C. The change in absorbance at 340 nm was monitored, and enzyme activity was quantitated by using $\Delta\epsilon_{340nm}$ = 6200 M^{-1} cm^{-1}.

GAR transformylase was assayed by a spectrophotometric assay with 10-formyl-5,8-dideazafolate as the formyl donor. Solutions of 0.25 mM (α + β) GAR, 0.01 mM 10-formyl-5,8-dideazafolate in 50 mM potassium phosphate, and 25% glycerol (pH 7.5) were incubated for 10 min at 37 °C. Assays were started with enzyme, and the reaction was monitored by following the increase in absorbance at 254 nm. GAR transformylase activity was quantitated with $\Delta\epsilon_{254nm}$ = 12 100 M^{-1} cm^{-1} (Smith et al., 1981). An alternative assay employed 0.2 mM GAR in 37.5 mM HEPES and 25% glycerol (pH 7.7), and the reaction was monitored at 295 nm ($\Delta\epsilon_{295nm}$ = 18 900 M^{-1} cm^{-1}).

AIR synthetase was assayed by a modification of the Bratton–Marshall reaction (Bratton & Marshall, 1939). Reaction mixtures in a final volume of 0.3 mL typically contained 50 mM HEPES (pH 7.7), 20 mM $MgCl_2$, 150 mM KCl, 0.1 mM FGAM, and 2.0 mM ATP. The reactions, incubated at 37 °C, were started by the addition of enzyme, and at varying time intervals the reaction mixture (0.3 mL) was quenched with 0.1 mL of 1.33 M potassium phosphate in 20% (w/v) trichloroacetic acid (pH 1.4). The mixture was then centrifuged (2000*g*, 1 min) and 0.1 mL of 0.1% (w/v) ammonium nitrate was added. After 3 min, 0.1 mL of 0.5% (w/v) ammonium sulfamate was added. After an additional 1 min, 0.025 mL of 0.25% (w/v) 1-*N*-naphthylethylenediamine dihydrochloride was added, and the absorbance at 500 nm ($\Delta\epsilon_{500nm}$ = 24 600 M^{-1} cm^{-1}) was measured after 10 min.

AIR carboxylase was assayed by the procedure of Patey & Shaw (1973). FGAR amidotransferase was assayed as previously described (Flaks & Lukens, 1963).

Protein Purification. A summary of the protein purification initially designed to obtain AIR synthetase is given in Table I. The last entry lists the three activities obtained from GAR transformylase purified by the method of Young et al. (1984). GAR synthetase has been purified as a fragment by the procedure of Murray and Stubbe[5] and had a specific activity

[3] J. L. Schrimsher and J. Stubbe, unpublished results.
[4] F. J. Schendel and J. Stubbe, unpublished results.
[5] M. V. Murray and J. Stubbe, unpublished results.

Table I: Purification of AIR Synthetase, GAR Synthetase, and GAR Transformylase Activities from the 60% Ammonium Sulfate Fraction of Chicken Liver

purification step	enzyme[a]	sp act.[b] (μmol min^{-1} mg^{-1})	rel sp act. (AS:GS: GT)
Sephadex G-25[c]	AS	0.0017	1:9.1:2.2
	GS	0.0154	
	GT	0.0037	
DEAE-cellulose	AS	0.0057	1:10.1:2.5
	GS	0.0575	
	GT	0.0141	
hydroxylapatite	AS	0.022	1:8.1:2.0
	GS	0.178	
	GT	0.045	
ATP–agarose	AS	0.19	1:10.8:2.0
	GS	2.05	
	GT	0.39	
Sephadex G-200	AS	0.41	1:9.8:2.0
	GS	4.01	
	GT	0.84	
10-CHO-5,8-dideaza-folate–Sepharose[d]	AS	0.32	1:13.0:3.0
	GS	4.15	
	GT	0.95	

[a] Abbreviations: AS, AIR synthetase; GS, GAR synthetase; GT, GAR transformylase. [b] Protein determinations were made by the method of Lowry et al. (1951). GAR synthetase was assayed by the [^{14}C]glycine assay. GAR transformylase activity was monitored at 254 nm with 10-CHO-5,8-dideazafolate as the formyl donor. [c] Purification procedure designed to isolate AS. [d] Purification procedure designed to isolate GT.

of 7 μmol min^{-1} mg^{-1}. This protein possessed neither AIR synthetase nor GAR transformylase activity.

Results and Discussion

The evidence for the presence of GAR synthetase, GAR transformylase, and AIR synthetase activities on a single multifunctional protein can be summarized as follows: (1) the three activities copurify in the same ratio through five fractionation steps; (2) an independent two-step chromatographic procedure that employs 10-formyl-5,8-dideazafolate (a folate analogue that can serve as cofactor for GAR transformylase) rather than ATP as the affinity ligand yields a protein that possesses the same values and ratio of the three activities (Table I, last entry); (3) a further purification protocol using ammonium sulfate precipitation plus hydroxylapatite, AICAR–Sepharose, and Cibacron Blue–agarose chromatography (Young et al., 1984) also yielded a protein with the same values and ratios of the three activities; (4) an antibody raised to murine GAR transformylase cross-reacts with the protein isolated from either procedure (1 or 2) in Western blots, and antibody raised to chicken GAR transformylase immunoprecipitates 98% of the three activities; and (5) limited chymotryptic digestion cleaves the protein into two fragments, one of which possesses the GAR synthetase activity and the other GAR transformylase activity.

The data in support of statements 1 and 2 are listed in Table I. It is highly improbable that all three of the protocols mentioned in the previous paragraph would give the same exact mixture of copurifying proteins with identical subunit molecular weights, especially since each protocol involves affinity chromatography fractionations. A Western blot (Figure 1) identified two bands (about 110000 and 55000) in the multifunctional protein samples obtained by either procedure and a single band (55000) in the GAR synthetase sample. In earlier work the protein associated with GAR transformylase activity was seen to undergo proteolytic cleavage to two species

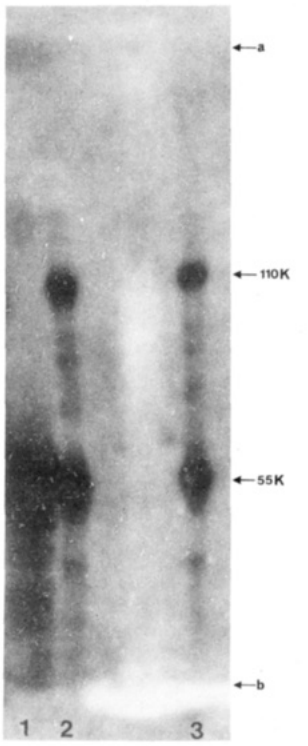

FIGURE 1: Immunoidentification of chicken liver GAR transformylase, GAR synthetase, and AIR synthetase by Western blot analysis. Western blot was performed as described under Methods. The blot was probed with 150 μL of crude serum from a rabbit immunized with murine GAR transformylase and 1.7 μCi of ^{125}I-labeled protein A with a specific radioactivity of 6 μCi/μg. The samples that appear are (lane 1) 51 μg of GAR synthetase, (lane 2) 1.4 μg of AIR synthetase, and (lane 3) 1.3 μg of GAR transformylase. a indicates the top of the gel and b indicates the electrophoretic front.

Table II: Separation of GAR Transformylase and GAR Synthetase after Partial Digestion by Chymotrypsin

fractions	GAR synthetase act. (μmol of GAR/10 min)[a]	GAR transformylase act. (μmol of FGAR/10 min)[b]
control		
first supernatant	0.0197	0.132
KP_i wash	0.0433	0.159
2 M urea eluate	0.121	2.55
% recovered	123	96
	(0.184/0.150[c] × 100)	(2.84/2.96[c] × 100)
digest		
first supernatant	0.0783	0.385
KP_i wash	0.0698	0.114
2 M urea eluate	0.0202	1.69
% recovered	113	74
	(0.168/0.150[c] × 100)	(2.14/2.96[c] × 100)

[a] GAR synthetase activity was monitored by coupling ADP production to NADH oxidation with the pyruvate kinase/lactate dehydrogenase couple as described under Methods. [b] GAR transformylase activity was monitored at 295 nm with 10-CHO-5,8-dideazafolate as the formyl donor as described under Methods. [c] Total activity in the stock sample.

of ca. 50000–55000 daltons (Young et al., 1984) from a single form of 110000 daltons. The 110000-dalton protein upon assay showed no FGAR amidotransferase, AIR carboxylase, or aminoimidazolesuccinocarboxamide ribonucleotide synthetase activities.

GAR synthetase isolated by an independent procedure[5] was shown to have a specific activity of 7 μmol min^{-1} mg^{-1} and a molecular weight of 55000 by SDS gel electrophoresis and 49000 by Sephadex chromatography. This protein possessed neither AIR synthetase nor GAR transformylase activity. However, this protein was immunoprecipitated by antibody

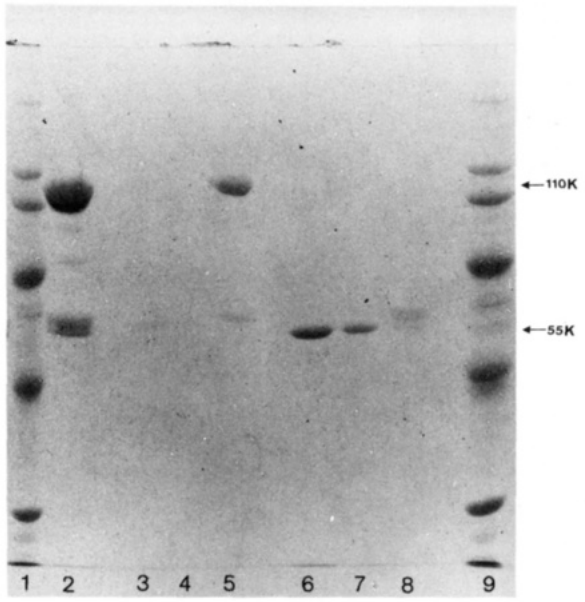

FIGURE 2: Partial chymotryptic digestion of GAR transformylase. Chicken GAR transformylase was subjected to partial chymotryptic cleavage. The fragments were separated by chromatography on 10-formyl-5,8-dideazafolate–Sepharose. Control and digested samples were subjected to electrophoresis on an 8.75% polyacrylamide gel in the presence of SDS. Lanes 1 and 9 contain marker proteins. Lane 2 contains 45 μg of GAR transformylase, as isolated by the purification protocol of Young et al. (1984). Lanes 3–5 contain the control samples, and lanes 6–8 contain the samples resulting from exposure to chymotrypsin. Lanes 3 and 6 contain the first supernatants from the batchwise adsorption of enzyme onto affinity resin. Lanes 4 and 7 contain the potassium phosphate washes of the resin, and lanes 5 and 8 contain the urea eluates from the resin. The amounts of protein applied to each lane are as follows: lane 3, 1 μg; lane 4, none; lane 5, 21 μg; lane 6, 10 μg; lane 7, 5 μg; lane 8, 6 μg. The anode is at the bottom of the gel.

prepared to GAR transformylase. Apparently, this purification procedure isolates the clipped domain which is responsible for GAR synthesis.

The purified multifunctional protein (which had been isolated by chromatography on 10-formyl-5,8-dideazafolate–Sepharose) was subjected to partial digestion with chymotrypsin, and the resulting fragments were separated by batchwise elution from 10-formyl-5,8-dideazafolate–Sepharose. It must be pointed out that when the deazafolate resin is used in the enzyme isolation procedure, there is 100% retention by the column of GAR synthetase activity; absolutely no activity is found in filtrates or washes of the affinity resin. The data from the proteolytic digest are listed in Table II. In the control 66% of GAR synthetase and 90% of GAR transformylase activities were found in the 2 M urea elution. This fraction was seen to consist of a single protein of 110 000 daltons after analysis by SDS gel electrophoresis (lane 5, Figure 2). In the digest 88% of GAR synthetase and 23% of GAR transformylase activities did not bind to the resin. These fractions consist of a protein of 55 000 daltons (lanes 6 and 7, Figure 2). However, 77% of the GAR transformylase activity bound to the resin and was eluted by 2 M urea, appearing as a species of ca. 64 000 daltons (lane 8, Figure 2).

The separation of GAR synthetase and GAR transformylase by proteolytic cleavage further demonstrates that copurification results from their presence on a single polypeptide. It is very unlikely that cleavage would be as selective for a protein mixture. In conclusion, there are at least four multifunctional proteins associated with de novo IMP biosynthesis: a trifunctional enzyme that possesses the 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, and 10-formyltetrahydrofolate synthetase activities, a bifunctional enzyme that exhibits aminoimidazolecarboxamide ribonucleotide transformylase and IMP cyclohydrolase activities (Benkovic, 1984), a bifunctional enzyme that possesses aminoimidazole ribonucleotide carboxylase and aminoimidazolesuccinocarboxamide ribonucleotide synthetase activities (Patey & Shaw, 1973), and the novel one described herein.

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