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Isolation of a Multifunctional Protein with Aminoimidazole Ribonucleotide Synthetase, Glycinamide Ribonucleotide Synthetase, and Glycinamide Ribonucleotide Transformylase Activities: Characterization of Aminoimidazole Ribonucleotide Synthetase†

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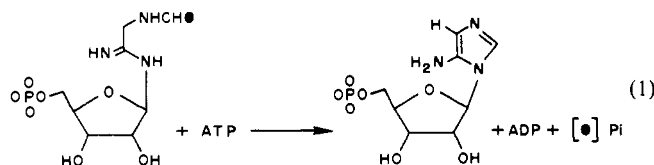
ABSTRACT: 5-Aminoimidazole ribonucleotide (AIR) synthetase, glycinamide ribonucleotide (GAR) synthetase, and GAR transformylase activities from chicken liver exist on a single polypeptide of M_r 110 000 [Daubner, C. S., Schrimsher, J. L., Schendel, F. J., Young, M., Henikoff, S., Patterson, D., Stubbe, J., & Benkovic, S. J. (1985) *Biochemistry 24*, 7059-7062]. Details of copurification of these three activities through four chromatographic steps are reported. The ratios of these activities remain constant throughout the purification. AIR synthetase has an absolute requirement for K^+ for activity and under these conditions has apparent molecular weights of 330 000, determined by Sephadex G-200 chromatography, and 133 000, determined by sucrose density gradient ultracentrifugation. Incubation of ^{18}O-labeled formylglycinamidine ribonucleotide (FGAM) with AIR synthetase results in stoichiometric production of AIR, ADP, and $[^{18}O]P_i$. NMR spectra of β-FGAM and β-AIR are reported.

In 1963, French and Buchanan (Flaks & Lukens, 1963) reported a 10-fold purification and characterization of aminoimidazole ribonucleotide synthetase from pigeon liver. This enzyme catalyzes the conversion of formylglycinamidine ribonucleotide (FGAM)[1] and ATP to aminoimidazole ribonucleotide (AIR), ADP, and P_i (eq 1). Since this early study,



no further progress has been reported toward isolation and purification of this fifth enzyme in the purine biosynthetic pathway. This is due in part to the difficulty in preparation of the substrate FGAM and in part to the reported instability of protein (Flaks & Lukens, 1963).

The recent results of an investigation between our laboratory and laboratories of Benkovic, Patterson, and Henikoff have established that AIR synthetase is part of a multifunctional protein which also contains two other activities of enzymes involved in purine biosynthesis, namely, glycinamide ribonucleotide (GAR) synthetase and glycinamide ribonucleotide transformylase (Daubner et al., 1985).

While GAR synthetase has not been previously isolated from chicken liver, GAR transformylase has been recently purified from this source by Caperelli et al. (1980) and more efficiently by Young et al. (1984) using a 10-formyl-5,8-dideazafolate affinity column. Thus, unknowingly, the isolation of GAR transformylase by Benkovic and his collaborators also con-

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[1] Abbreviations: AIR, 5-aminoimidazole ribonucleotide; FGAM, formylglycinamidine ribonucleotide; PMSF, phenylmethanesulfonyl fluoride; AIRs, 5-aminoimidazole ribonucleoside; GAR, glycinamide ribonucleotide; Tris, tris(hydroxymethyl)aminomethane; FGAR, formylglycinamide ribonucleotide; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; TEAB, triethylammonium bicarbonate; AICAR, 5-aminoimidazolecarboxamide ribonucleotide; ATPγS, adenosine 5'-O-(3-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide; Cl_3CCOOH, trichloroacetic acid; FID, free induction decay.

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stituted the first reported isolation of GAR synthetase and AIR synthetase.

Our laboratory has been interested in the mechanism of AIR synthetase and GAR synthetase, and hence our recent efforts have been directed toward isolating these activities. This paper reports the detailed isolation and purification to homogeneity of AIR synthetase and the copurification of GAR synthetase and GAR transformylase. The kinetic and physical properties of AIR synthetase are also reported. In addition, [^{18}O]FGAM prepared biosynthetically and characterized by NMR spectroscopy has been shown to transfer its oxygen quantitatively to P_i during the production of ADP and AIR (eq 1). FGAM and AIR have also been characterized by NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. 10-Formyl-5,8-dideazafolate was a generous gift of Mark Young and S. J. Benkovic (The Pennsylvania State University). GAR (a 1:1 mixture of α and β anomers) was synthesized according to the procedure of Chettur and Benkovic (1977). β-FGAM was prepared as described by Daubner et al. (1985). [γ-^{32}P]ATP was synthesized according to the procedure of Selman and Selman-Reimer (1981). The [^{32}P]P_i used for [γ-^{32}P]ATP synthesis and [^{14}C]formate (51.5 mCi/mmol) were obtained from New England Nuclear Corp. [2-^{14}C]Glycine (52 mCi/mmol) was obtained from Amersham. [^{13}C]Formic acid (99.5 atom % ^{13}C) was purchased from MSD Isotopes. $H_2{}^{18}O$ (98.1 atom % ^{18}O) was obtained from Monsanto Research Corp. [^{13}C,$^{18}O_2$]Formate was synthesized by the procedure of Hermes et al. (1984). The ^{18}O content of the formate was established by ^{13}C NMR. ATP, D-ribose 5′-phosphate, bovine serum albumin, L-lactate dehydrogenase (rabbit muscle) (920 μmol min^{-1} mg^{-1}), pyruvate kinase (rabbit muscle) (355 μmol min^{-1} mg^{-1}), phosphoenolpyruvate, molecular weight standards for gel filtration and gel electrophoresis, ATP–agarose (linked through N-6 of the adenine ring by a six-carbon spacer, 1.4 μmol of ATP/mL of resin), Sephadex G-25 (fine), and DEAE-Sephadex A-25 were obtained from Sigma Chemical Co. Hydroxylapatite was purchased from Bio-Rad Laboratories. ATPγS was obtained from P-L biochemicals (Piscataway, NY). All other reagents used were of reagent grade or better unless otherwise specified. PCO 135 (purE$^-$) was obtained from the *Escherichia coli* Genetics Stock Center, Yale University, New Haven, CT. NMR spectra were recorded on either a Bruker 270-MHz spectrometer or a Nicolet 200-MHz broad-band spectrometer.

Protein Determination. Protein concentrations of the solutions were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Gel Filtration Column Calibration. The column of Sephadex G-200 was calibrated with blue dextran (average molecular weight of 2 × 10^6) and proteins of known molecular weight in order to obtain an estimate of the native molecular weight of the protein with GAR synthetase, AIR synthetase, and GAR transformylase activities. The proteins used were horse spleen ferritin, bovine liver catalase, rabbit muscle lactate dehydrogenase, and bovine serum albumin. The running buffer contained 100 mM potassium phosphate (pH 7.4) or 100 mM potassium phosphate (pH 7.4), 5% glycerol, 1 mM EDTA, and 5 mM β-mercaptoethanol. Protein was loaded onto the column at a concentration of 2–3 mg/mL.

Sucrose Density Gradient Ultracentrifugation. This technique was performed by the method of Martin and Ames (1961) in 5–20% sucrose gradients (4.8 mL total volume) in either 50 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol or 100 mM potassium phosphate (pH 7.5) and 10 mM β-mercaptoethanol. Gradients were poured at room temperature and allowed to equilibrate at 4 °C overnight. Centrifugation was performed on 0.1 mL of enzyme (2.0 mg/mL) at 4 °C in a Beckman Model L5-50 preparative ultracentrifuge equipped with an SW-50.1 rotor. Centrifugation at 40 000 rpm was carried out for varying time periods (5–20 h). Sedimentation coefficients ($s_{20,w}$) were determined by using lysozyme, egg albumin, yeast alcohol dehydrogenase, and catalase as standards in separate tubes centrifuged concurrently.

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

Purification of AIR Synthetase. The purification of the protein that contained GAR synthetase, GAR transformylase, and AIR synthetase activities was performed at 4 °C on fresh chicken liver. All buffers contained 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol in addition to the other components specified below, unless otherwise noted.

The liver was homogenized in 2.5 volumes of a buffer that contained 20 mM potassium phosphate (pH 7.4), 50 mM KCl, and 0.5 mM PMSF. The homogenate was centrifuged (18000*g*, 30 min), and the supernatant was filtered through cheesecloth. This solution was treated with 277 g/L ammonium sulfate (45% saturation), stirred for an additional 30 min, and centrifuged (18000*g*, 20 min). The supernatant was adjusted to 60% saturation in $(NH_4)_2SO_4$ (99 g/L), stirred 30 min, and centrifuged (18000*g*, 30 min). The precipitate was taken up in a minimum amount of buffer that contained 10 mM Tris-HCl (pH 8.2) and 100 mM KCl. This solution with 0.5 mM PMSF added could be stored at −80 °C for several months without significant loss of activity. Large amounts of fresh liver were routinely processed in this way, and the solution was stored in 200-mL aliquots for future purification. The solution from the 60% ammonium sulfate precipitation (138 mL) was subjected to gel filtration on a column of Sephadex G-25 (6 × 46 cm) equilibrated with buffer that contained 10 mM Tris-HCl (pH 8.2) and 10 mM KCl. The effluent that contained protein was collected and diluted with an equal volume of water that contained 10% (v/v) glycerol and 1 mM PMSF. When necessary, the pH was adjusted to 8.2 with 0.5 M KOH.

The diluted solution from the Sephadex G-25 column was applied (4–6 mL/min) to a column of DEAE-cellulose (Whatman DE-52, column volume of 2 mL/g of tissue homogenized) previously equilibrated with buffer that contained 10 mM Tris-HCl (pH 8.2) and 10 mM KCl. The column was then washed with the sample buffer until the absorbance (280 nm) of the effluent was less than 0.3 and developed with a linear gradient of KCl (10–120 mM) in the equilibration buffer. Fractions that contained the appropriate activities (determined by the assays described below) were pooled and applied to a column (4.0 × 9.5 cm) of hydroxylapatite previously equilibrated with a buffer that contained 20 mM potassium phosphate (pH 7.4) and 20 mM KCl. The column was then washed with the equilibration buffer until the absorbance of the effluent (280 nm) was less than 0.1 and developed with a 1200-mL linear gradient of potassium phosphate buffer (pH 7.4, 20–300 mM). Fractions of 15 mL were collected.

Fractions that contained the appropriate activities were pooled and concentrated to a volume of 10–20 mL by using an Amicon ultrafiltration apparatus with a PM30 membrane.

This concentrated solution was subjected to gel filtration (Sephadex G-25, 4.0 × 18 cm) in 10 mM Tris-HCl (pH 8.2) and 10 mM KCl as described for the 60% ammonium sulfate precipitate. The resulting protein solution was applied to a column (1.5 × 6 cm) of ATP–agarose (1 mL of column material per unit of AIR synthetase activity), and the column was washed with the 10 mM Tris-HCl (pH 8.2) and 10 mM KCl until no protein could be detected in the effluent (A_{280}). The column was then developed with 1 mM ATP in the same buffer. Fractions that contained the appropriate activities were pooled and concentrated to a volume of 5 mL by using an Amicon apparatus and a PM30 membrane.

The concentrated enzyme was applied to a column (2.5 × 113 cm) of Sephadex G-200 equilibrated with a buffer that contained 100 mM potassium phosphate, pH 7.4, and 5% (v/v) glycerol. The flow rate was maintained at 5–6 mL/h. Fractions that contained the enzymatic activities were concentrated to give a protein solution of 1–2 mg/mL and stored at −80 °C.

Enzyme Assays. One unit of activity is defined as the amount of enzyme required to produce 1 μmol/min of product. All assays were performed at 37 °C unless otherwise noted.

AIR Synthetase Assay. AIR synthetase was quantitated by one of three methods: production of [^{32}P]P$_i$, ADP, or AIR. (1) P_i production was quantitated by using scintillation counting. A typical reaction mixture contained in a final volume of 60 μL 50 mM HEPES (pH 7.7), 20 mM $MgCl_2$, 150 mM KCl, 0.1 mM β-FGAM, 2.0 mM [γ-^{32}P]ATP (10^7 cpm/μmol), and (0.2–1.0) × 10^{-4} unit of AIR synthetase. At 2-min intervals, 10-μL aliquots and 10 μmol of carrier P_i were quenched with 2.0 mL of acid molybdate solution [$(NH_4)_6Mo_7O_{24}\cdot 7H_2O$ (1% w/v) in 0.8 M perchloric acid]. Inorganic phosphate, [^{32}P]P$_i$, was immediately extracted into 2 mL of isobutyl alcohol/benzene (1:1 v/v), and 1 mL of this solution was subjected to scintillation counting. (2) ADP formation was quantitated by a coupled reaction procedure. A typical reaction mixture identical with that described above contained in addition 3.5 units of pyruvate kinase, 4.7 units of lactate dehydrogenase, 1.0 mM phosphoenolpyruvate, and 0.2 mM NADH (final volume of 1 mL). The initial velocities were determined spectrophotometrically by observing a decrease in A_{340nm} (ϵ = 6200 M^{-1} cm^{-1}). (3) Production of AIR was quantitated through a modification of the Bratton–Marshall reaction (Bratton & Marshall, 1939). At varying time intervals, the reaction mixture identical with that described above in assay 1 (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) to remove precipitated protein, and 0.1 mL of 0.1% (w/v) ammonium nitrite was added. After 3 min, 0.1 mL of 0.5% (w/v) ammonium sulfamate was added. After an additional minute, 0.025 mL of 0.25% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride was added, and the absorbance at 500 nm (ϵ = 24 600 M^{-1} cm^{-1}) was measured after 10 min.

GAR Synthetase Assay. A typical reaction mixture contained in a final volume of 0.5 mL 100 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 20 mM D-ribose 5-phosphate, 290 mM NH_4Cl, 1 mM ATP, 10 mM [^{14}C]glycine (1 × 10^5 cpm/μmol), and 0.001–0.005 unit of GAR synthetase. At 2-min time intervals, including a zero time point, 100 μL of the reaction mixture was quenched with 15 μL of 30% (w/v) trichloroacetic acid and added to a column (0.5 × 4 cm) of Dowex 50W X8 (equilibrated in 50 mM ammonium formate, pH 3.3) which was subsequently washed with 2.5 mL of 50 mM ammonium formate (pH 3.3). The effluent that contained GAR was collected, and the amount of GAR was quantitated by scintillation counting.

GAR Transformylase. The reaction mixture contained in a final volume of 1 mL 0.25 mM α/β-GAR, 0.011 mM 10-formyl-5,8-dideazafolate, 20 mM HEPES (pH 7.5), and 0.0175–0.035 unit of GAR transformylase. The reaction mixture incubated at 22 °C was initiated by the addition of enzyme, and the reaction was monitored by the disappearance of the 10-formyl-5,8-dideazafolate (ϵ_{254} = 23 500 M^{-1} cm^{-1}).

Characterization of β-FGAM. β-FGAM was synthesized biosynthetically by modifications of the procedure of Levenberg and Buchanan (1957b) and Daubner et al. (1985): NMR [D_2O, pD 6.0, and HOD (δ 4.4)] δ 7.85 (1 H, s, formyl proton), 4.88 (1 H, d, *J* = 5 Hz, 1′). The 2′-, 3′-, 4′-, and 5′-hydrogens of the sugar moiety and the CH_2 of the glycinamidine side chain appeared between δ 3.48 and δ 4.07 (Figure 4).

Isolation of 5-Aminoimidazole Riboside. The 5-aminoimidazole riboside was isolated by a modification of the procedure of Estramareix and Therisod (1984). The *E. coli* mutant PCO 135 (*purE*$^-$) was grown on minimal media containing 6 g of KH_2PO_4, 14 g of K_2HPO_4, 1 g of sodium chloride, 0.4 g of magnesium sulfate (heptahydrate), and 2 g of ammonium sulfate (all per liter), pH 7.2, supplemented with 10 mL of 20% glucose (w/v), 5 mL of 0.4% leucine (w/v), 5 mL of 0.6% threonine (w/v), and 3 mL of 0.5% hypoxanthine (w/v). The cells were harvested in late log phase by centrifugation at 8000*g*, with a yield of 2–3 g of cells per liter of culture. For the synthesis of AIRs, approximately 10 g of cells was resuspended in 1 L of the minimal salts supplemented with 10 mL of 20% glucose, 10 mL of 100 mM glycine, and 10 mL of 100 mM sodium formate. The culture was shaken at 37 °C for 3 h, and the cells were separated from the media by centrifugation (the cells could be used 4–5 times for the synthesis of AIRs). The pH of the media was adjusted to 5.0 with 3 M HCl and then applied to a Dowex 50W X8 (ammonium form) column (4 × 13 cm). The column was washed with 2 volumes of water, and the AIRs was eluted with 20 mM ammonium hydroxide. The fractions containing AIRs were identified by the Bratton–Marshall assay for diazotizable amines (Bratton & Marshall, 1939), pooled, evaporated to dryness in vacuo, and stored at −20 °C. A typical yield is 140 μmol: ^{1}H NMR (Me_2SO-d_6, δ 2.46 Me_2SO) δ 3.53 (m, 2 H, 5′a,b), 3.79 (m, 1 H, 4′), 4.00 (t, 1 H, *J* = 4.5 Hz, 3′), 4.22 (t, 1 H, *J* = 6.0 Hz, 2′), 5.38 (d, 1 H, *J* = 6.0 Hz, 1′), 6.01 (s, 1 H C-4 proton of imidazole), 7.26 (s, 1 H, C-2 proton of imidazole ring); ^{13}C NMR (Me_2SO-d_6, δ 40.1) δ 61.25 (5′), 70.08, 73.43 (2′ and 3′), 84.89 (4′), 87.56 (1′), 110.57 (C-4 of imidazole ring), 129.92 (C-2 of imidazole ring), 137.39 (C-5 imidazole ring).

Synthesis of 5′-Aminoimidazole Ribonucleotide. AIRs was phosphorylated by the method of Yoshikawa et al. (1967). To 274 μmol of AIRs, which had been dried over P_2O_5 under vacuum, was added 2.7 mL of freshly distilled triethyl phosphate. The reaction was cooled to 0 °C, and 0.27 mL (2.8 mmol) of $POCl_3$ (freshly distilled) was added, and the reaction was allowed to proceed for 2 h at 0 °C. The black solution was added to 100 mL of anhydrous ether and the precipitate collected by centrifugation. The pellet was washed 2 times with 40 mL of ether, resuspended in 40 mL of ether, and transferred to a 100-mL round-bottom flask. The ether was removed in vacuo, and the residue was redissolved in 200 mL of cold water with the pH of solution maintained at 7 by the addition of 1 N sodium hydroxide until the pH stopped dropping. The solution was diluted to 300 mL with H_2O and

applied to a DEAE-Sephadex A-25 column (2.5 × 15 cm). The column was eluted with a 800-mL linear gradient from 0 to 400 mM triethylammonium bicarbonate (pH 7.8). Fractions were assayed for AIR by the Bratton–Marshall assay, pooled, and evaporated to dryness in vacuo, giving 42 μmol (15%) yield: ^{1}H NMR [D_2O, pD 5.2, HOD (δ 4.40)] δ 3.67 (m, 2 H, 5′a,b), 3.94 (m, 1 H, 4′), 4.01 (t, J = 4.4 Hz, 1 H, 3′), 4.16 (t, J = 4.4 Hz, 1 H, 2′), 5.45 (d, J = 4.4 Hz, 1 H, 1′), 6.33 (s, 1 H, exchangeable, C-4 proton of imidazole ring), 8.19 (s, 1 H, C-2 proton of imidazole ring); ^{13}C NMR (D_2O, pD 6.0, external standard dioxane δ 66.5) δ 63.30 (5′), 70.09 (2′), 73.25 (3′), 83.89 (J_{CCP} = 8.5 Hz, 4′), 87.17 (1′), 110.59 (C-4 of imidazole), 131.02 (C-2 of imidazole), quaternary carbon of the imidazole ring was not observed.

Stoichiometry of Product Production. Three identical reaction mixtures were monitored for the production of P_i, ADP, and AIR as described under enzyme assays for AIR synthetase.

pH Dependence of AIR Synthetase. Each reaction mixture contained in a final volume of 1 mL 20 mM $MgCl_2$, 10 mM ATP, 0.06 mM FGAM, 80 mM KCl, 50 mM Tris-HCl (pH 8–9) or 50 mM HEPES (pH 7–8), and 0.005 unit of AIR synthetase. At various times, 0.25-mL aliquots were quenched, and the amount of AIR produced was determined by Bratton–Marshall assay as described above.

Metal Dependence. To determine optimal K^+ concentration, the reaction mixture was identical with that described above except 50 mM HEPES (pH 7.7) was utilized as buffer and the amount of KCl was varied from 0 to 200 mM. To determine the optimal Mg^{2+} concentration, the reaction mixture contained 1.6 mM MgATP, 50 mM HEPES (pH 7.7), 150 mM KCl, 0.08 mM FGAM, and 0.005 unit of AIR synthetase in a final volume of 1 mL. The concentration of $MgCl_2$ was varied between 0 and 20 mM. The amount of AIR produced was determined by the Bratton–Marshall assay as described above.

Kinetic Analysis. All kinetic studies were conducted in the presence of 50 mM HEPES (pH 7.7), 20 mM $MgCl_2$, and 150 mM KCl. The values of the Michaelis constant for FGAM and ATP were determined by varying the concentration of the substrate from $^1/_5$th to 5 times their respective K_m values. The data were fit to a nonlinear regression analysis computer program to eq 2 or 3 to determine the best steady-state kinetic

$$v = \frac{VAB}{K_AB + K_BA + AB + K_{ia}K_B} \quad (2)$$

$$v = \frac{VAB}{K_AB + K_BA + AB} \quad (3)$$

model. Product inhibition studies with ADP, P_i, and AIR were analyzed by using BASIC versions of the computer programs of Cleland (1979). The data from the inhibition studies were fit to eq 4–6 for instances of competitive, noncompetitive, or uncompetitive inhibition, respectively.

$$v = \frac{VA}{K_A(1 + I/K_{is}) + A} \quad (4)$$

$$v = \frac{VA}{K_A(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (5)$$

$$v = \frac{VA}{K_A + A(1 + I/K_{ii})} \quad (6)$$

ATPγS as Substrate for Chicken Liver AIR Synthetase. The reaction mixture contained in a final volume of 1.3 mL 0.1 mM FGAM, 50 mM HEPES (pH 7.7), 150 mM KCl, and 20 mM $MgCl_2$ in addition to variable amounts of AIR synthetase and ATPγS. At varying time intervals, 0.2-mL aliquots were removed and analyzed for AIR as described above. In addition, a similar reaction mixture was analyzed for thiophosphate production by the procedure of Webb and Trentham (1980). At varying times, 0.3-mL aliquots were applied to a column (0.5 × 2.0 cm) of DEAE-Sephadex (A-25). The column was then washed with 5 mL of water, and the thiophosphate product eluted with 5 mL of 0.20 M triethylammonium bicarbonate (pH 7.5). The effluent that contained the thiophosphate was evaporated to dryness in vacuo, and the residue was quantitated by using 5,5′-dithiobis(2-nitrobenzoic acid).

Biosynthesis of GAR. GAR was synthesized by the procedure of Chettur and Benkovic (1977) or by the biosynthetic method described below. In a 50-mL Erlenmeyer flask were placed 800 μmol of ribose 5-phosphate, 400 μmol of ATP, 200 μmol of glycine, 2.5 μCi of [^{14}C]glycine, 1.6 mmol of $MgCl_2$, and 10 mL of Tris base saturated with NH_4Cl (pH 8.5). Partially purified GAR synthetase,[4] 20 units, in 10 mL of Tris-HCl (pH 7.5) was added, and the reaction was incubated at 37 °C for 1 h. The protein was precipitated by the addition of 1 mL of 60% Cl_3CCOOH and removed by centrifugation. The supernatant was diluted to 500 mL, the pH adjusted to 8.0 with 3 N KOH, and applied to a DEAE-Sephadex A-25 column (4 × 30 cm). The column was washed with 2 volumes of water, and the GAR eluted with a 2-L linear gradient from 0 to 300 mM TEAB. The fractions which contained GAR eluted at 125 mM TEAB in the gradient and were pooled, concentrated to dryness in vacuo, and redissolved in 10 mM ammonium acetate buffer (pH 4.5). The GAR was passed through a Dowex-1 X8 column (2.5 × 20 cm), equilibrated with 10 mM ammonium acetate (pH 4.5), concentrated in vacuo, and adjusted to pH 8.5 with 3 N KOH, and 300 μmol of barium acetate was added. Five volumes of absolute ethanol was added, and the solution was placed at −20 °C for 4 h. The barium salt of GAR was collected by centrifugation and dried over P_2O_5 under vacuum to yield 133 μmol of β-GAR/α-GAR (82:18): ^{1}H NMR [D_2O, pD 6.0, β-anomer) δ 3.44 (s, 2 H, glycine CH_2), 3.53 (m, 3 H, 4′ and 5′), 3.74 (t, 1 H, J = 5.2 Hz, 3′), 3.86 (t, 1 H, J = 5.2 Hz, 2′), 5.09 (d, 1 H, J = 5.3 Hz, 1′).

Preparation of Formate Incorporation Enzymes. Cells from the *E. coli* mutant PCO 135 (*purE*$^-$) (5 g), which had been used to synthesize AIRs, were resuspended in 2 volumes of 20 mM potassium phosphate (pH 7.4) and broken in a French pressure cell at a pressure of 16000 psi. The broken cells were centrifuged at 27000g for 30 min, and the supernatant was applied to a Sephadex G-25 column (4 × 30 cm) equilibrated in 10 mM Tris-HCl (pH 7.5). The protein peak was collected and used in the biosynthesis of FGAR from GAR and sodium formate (15 mL, ≈60 mg/mL).

Biosynthesis of [^{16}O,^{18}O,^{13}C]FGAR. Into a 25-mL conical flask was placed 125 μmol of ATP, 250 μmol of $MgCl_2$, 20 μmol of β-GAR (or 40 μmol of α/β-GAR), 0.10 mM azaserine, 200 μmol of sodium [^{13}C/^{18}O]formate (^{18}O enrichment > 95% by ^{13}C NMR), [^{14}C]formate (9.0 μCi), and 200 μmol of sodium [^{13}C]formate in 5 mL of 350 mM Tris-HCl (pH 8.0). Formate incorporation enzymes, 600 mg, in a volume of 10 mL of 10 mM Tris-HCl (pH 7.5) were added, and the reaction mixture was incubated at 37 °C for 1 h. The protein was precipitated by Cl_3CCOOH (0.5 mL, 60%) and removed by centrifugation. The supernatant was diluted to 300 mL with water, with the pH adjusted to 8.0 with 3 N KOH, and applied to a DEAE-Sephadex A-25 column (2.5 × 15 cm). The column was washed with 2 volumes of water and eluted

Table I: Chicken Liver AIR Synthetase Purification

step	protein (mg/mL)	volume (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	x-fold purification	recovery (%)	related act., AS:GS:CT[b]
crude[a]	30.6	465	14257	20.7	0.0014		100	
$(NH_4)_2SO_4$, 45%	20.9	432	9020	18.5	0.0020	1.4	75	
$(NH_4)_2SO_4$, 60%	39.4	142	5595	12.4	0.0022	1.5	60	1:9.1:2.2
DE-52	1.20	650	777	11.2	0.014	9.9	54	1:10.1:2.5
hydroxylapatite	0.63	250	158	7.6	0.048	33.0	37	1:8.1:2.0
ATP-agarose	0.97	33	31.9	6.9	0.21	149	33	1:10.8:2.0
G-200	1.30	10	12.9	5.6	0.43	299	27	1:9.8:2.0

[a] 200 g of fresh chicken liver was utilized. [b] AS = AIR synthetase, GS = GAR synthetase, and GT = GAR transformylase.

with a 1-L linear gradient from 0 to 300 mM TEAB. The FGAR-containing fractions eluted at 200 mM TEAB and were located by liquid scintillation counting, pooled, and concentrated to dryness in vacuo. The FGAR was redissolved in 15 ml of water, the pH was adjusted to 8.5 with 3 N KOH, 200 μmol of barium bromide was added, and the barium phosphate which formed was removed by centrifugation. The pH of the supernatant was readjusted to 8.5, 5 volumes of absolute ethanol added, and the solution placed at −20 °C for 4 h. The precipitated FGAR was collected by centrifugation and dried over P_2O_5 under vacuum, and the Ba^{2+} salt was exchanged for Na^+ by passage through a Dowex 50W X8 column (1.5 × 3 cm, sodium form), yield 17.2 μmol. The ^{18}O content of the FGAR was determined by ^{13}C NMR at 125 MHz using broad-band proton decoupling, a sweep width of ±4700 Hz (quadrature phase detection), a 90° pulse angle, and a 64K data block with an acquisition time of 3.47 s. To enhance resolution, the spectrum was recorded at 37 °C, and exponential multiplication with a line-broadening factor of −0.02 Hz was applied to the FID before Fourier transformation: ^1H NMR (D_2O, pD 6.0) δ 7.89 (d, 1 H, $J_{^{13}CH}$ = 198.8 Hz, formyl proton), 5.15 (d, 1 H, J = 5.3 Hz, 1′).

Synthesis of FGAM from FGAR. The labeled [^{14}C,^{13}C,^{18}O]FGAR (5 × 10^4 cpm/μmol) was converted to FGAM by the procedure of Daubner et al. (1985) except that the FGAM was located by liquid scintillation counting instead of phosphate assay.

^{18}O Transfer Experiment. To a 5-mm NMR tube containing 20 μmol of ATP, 40 μmol of $Mg(OAc)_2$, 150 μmol of KCl, and 20 μmol of HEPES (pH 7.8) in a final volume of 1 mL (30% D_2O) was added 0.8 unit of AIR synthetase. An 80-MHz ^{31}P NMR spectrum was recorded, then 5 μmol of [^{18}O,^{16}O]FGAM was added, and the NMR tube was incubated at 37 °C for 30 min. When the reaction was complete as determined by ^{31}P NMR, the reaction was applied to a DEAE-Sephadex A-25 column (0.4 × 2 cm), and the column was washed with 5 mL of H_2O, the P_i eluted with 5 mL of 250 mM TEAB, and concentrated to dryness in vacuo. The ^{31}P NMR spectrum was taken at 80 MHz using D_2O as the lock solvent. A ±400-Hz sweep width (quadrature phase detection), a 90° pulse angle, and a 8K data block were used. The acquisition time was 3.4 s, and the pulse delay was 1 s. To enhance resolution, exponential multiplication with a line-broadening factor of 0.1 Hz was applied to the FID before Fourier transformation.

Results

Purification. AIR synthetase has been purified 300-fold to homogeneity from a 60% ammonium sulfate precipitate of fresh chicken liver as indicated in Table I. In addition, both GAR synthetase and GAR transformylase were found to coelute with AIR synthetase in each of the four chromatographic procedures (Figures 1 and 2), and the relative specific activities of the three proteins remained constant throughout

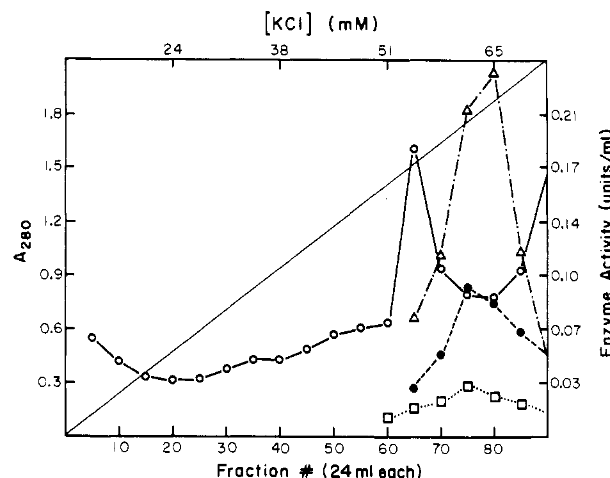


FIGURE 1: DE-52 column profile (5.0 × 32 cm). Column eluted with a 4-L linear gradient (10–120 mM KCl) in 10 mM Tris-HCl (pH 8.2). Fractions of 22 mL were collected. (○) Protein A_{280}; (△) GAR synthetase activity; (●) AIR synthetase activity; (□) GAR transformylase activity.

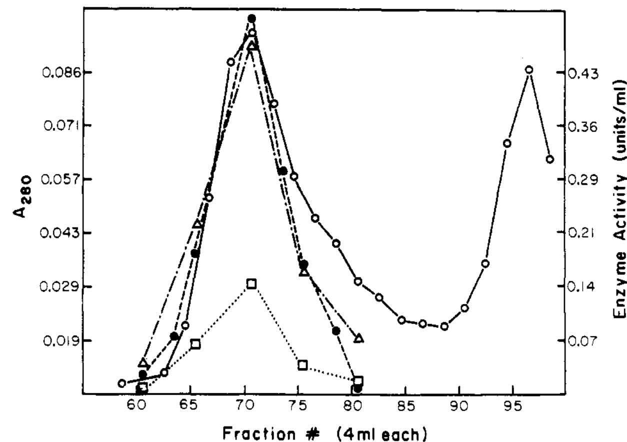


FIGURE 2: Sephadex G-200 column profile (2.5 × 118 cm). Column eluted with 100 mM KCl and 50 mM HEPES (pH 7.7). Fractions of 4 mL were collected. (○) Protein A_{280}; (△) GAR synthetase activity; (●) AIR synthetase activity; (□) GAR transformylase activity.

the purification (Table I). The enzyme obtained by this procedure was stable for many weeks when stored at −20 °C; however, on repeated freeze–thawing, activity was lost due to the precipitation of protein. The enzyme is therefore routinely stored (1–2 mg/mL) in small aliquots at −80 °C.

Native and Subunit Molecular Weight. Gel filtration on Sephadex G-200 of AIR synthetase indicates that the protein under a wide variety of conditions, including 100 mM potassium phosphate (pH 7.5) ± glycerol, gave an apparent molecular weight of 330000 when compared with standards. These results contrast with those obtained by using sucrose density gradient ultracentrifugation which under a variety of conditions gave an apparent native molecular weight of

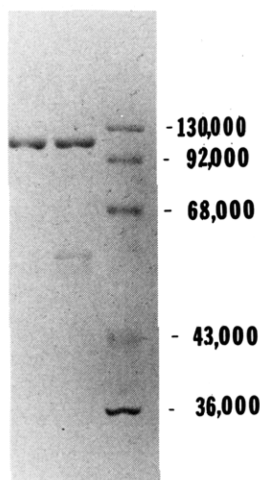


FIGURE 3: SDS gel electrophoresis of AIR synthetase. Left lane, purified by isolation procedure described in Table I; middle lane, purified by method of Young et al. (1984); right lane, molecular weight standards.

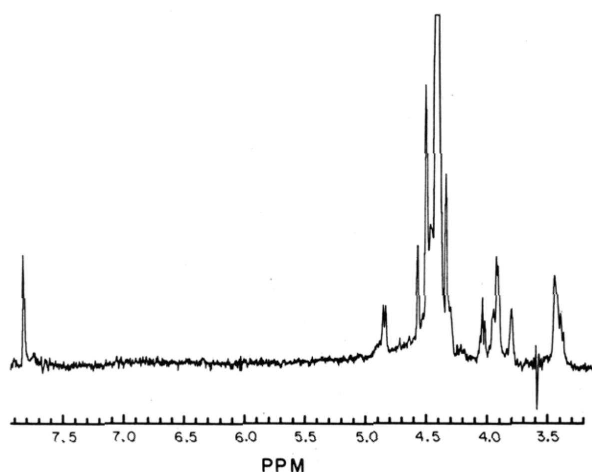


FIGURE 4: NMR spectrum of β-FGAM prepared biosynthetically.

133 000. Furthermore, SDS gel electrophoresis (7.5% acrylamide) with known molecular weight standards gave one major protein band (90%) corresponding to a subunit molecular weight of 110 000 and a minor protein band corresponding to M_r 55 000[2] (Figure 3). Thus, the subunit stoichiometry of AIR synthetase, under conditions which produce maximum specific activity, needs to be further investigated.

Characterization of FGAM and AIR. Presently no chemical methods are available for the synthesis of pure FGAM and AIR. Therefore, FGAM was prepared biosynthetically with FGAM synthetase[3] and isolated by anion-exchange chromatography (Daubner et al., 1985). The NMR spectrum indicated in Figure 4 shows a single anomeric proton at 4.9 ppm, consistent with biosynthetic production of only the β isomer. Aminoimidazole riboside was readily prepared biosynthetically by extensive modifications of the procedure of Estramareix and Therisod (1984). The nucleoside was then phosphorylated to give AIR, whose NMR spectrum is indicated in Figure 5. The major product (>90%) shows a single anomeric proton at 5.45 ppm, also consistent with biosynthetic production of the pure β isomer.

[2] Antibodies prepared to chicken liver GAR transformylase by Benkovic and his co-workers were cross-reactive with both protein bands, consistent with the interpretation that the smaller protein is a proteolytic breakdown product of the larger protein.

[3] Isolated by the procedure of Schendel and Stubbe with a specific activity of 0.20 μmol min^{-1} mg^{-1}.

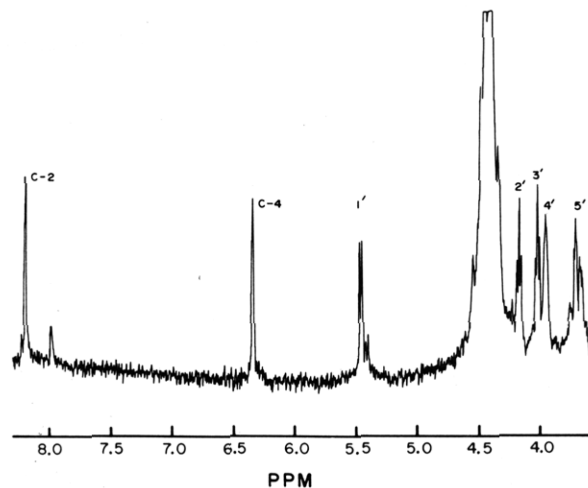


FIGURE 5: NMR spectrum of AIR prepared as described under Experimental Procedures. Resonances assigned by decoupling experiments.

Stoichiometry of Product Production. The estimation of the amount of AIR produced by AIR synthetase is based on the A_{500nm} of the chromophore produced as a result of the Bratton and Marshall (1939) assay for diazotizable amines. However, widely ranging values for this extinction coefficient have been reported. In 1957, Levenburg and Buchanan reported a value of 24 600 M^{-1} cm^{-1} for AIR produced biosynthetically (Levenburg & Buchanan, 1957a) while in 1966 Frank et al. reported a value of 17 000 M^{-1} cm^{-1} for material prepared synthetically. From the assays described under Experimental Procedures, products of the reaction catalyzed by AIR synthetase were found to be 1:1:1 (AIR/ADP/P_i) when a value of 24 800 (±1600) M^{-1} cm^{-1} was used as the extinction coefficient for this chromophore. This value is therefore in agreement with the original value reported by Levenburg and Buchanan.

Kinetics. Prior to investigation of the kinetic properties of AIR synthetase, the optimal conditions for enzyme turnover of FGAM to AIR were determined. The pH optimum was found to be 7.7 with the activity remaining 90% optimum between pH 7.4 and 8.1. These results are similar to the value of 7.8 reported for the partially purified pigeon liver enzyme (Flaks & Lukens, 1963). Both Mg^{2+} and K^+ were found to be essential for catalysis, with 20 mM Mg^+ giving optimal activity over a broad MgATP range (0.01–10.0 mM). At high concentrations of Mg^{2+}, greater than 20 mM, inhibition of AIR production was observed, and at low concentrations, less than 5.0 mM, substrate inhibition by FGAM was observed. Concentrations of KCl of 150 mM gave optimal activity under all conditions investigated. On the basis of these studies, all kinetic investigations described subsequently were performed in 50 mM HEPES (pH 7.7), 150 mM KCl, and 20 mM $MgCl_2$.

Initial velocity studies on the AIR synthetase reaction using β-FGAM and MgATP revealed a series of parallel lines as summarized in Figure 6. The kinetic constants derived from these studies were K_m = 0.078 mM for MgATP, K_m = 0.009 mM for β-FGAM, and V_{max} = 0.40 μmol min^{-1} mg^{-1}. Product inhibition patterns using P_i, ADP, and AIR were also investigated and gave results summarized in Table II. Unfortunately, the observed patterns and the irreversibility of the overall reaction do not allow a determination of the sequence of substrate binding or product release.

Transfer of ^{18}O from β-[^{18}O]FGAM to P_i. β-[^{16}O/^{18}O,^{13}C]FGAR was prepared biosynthetically (Experimental

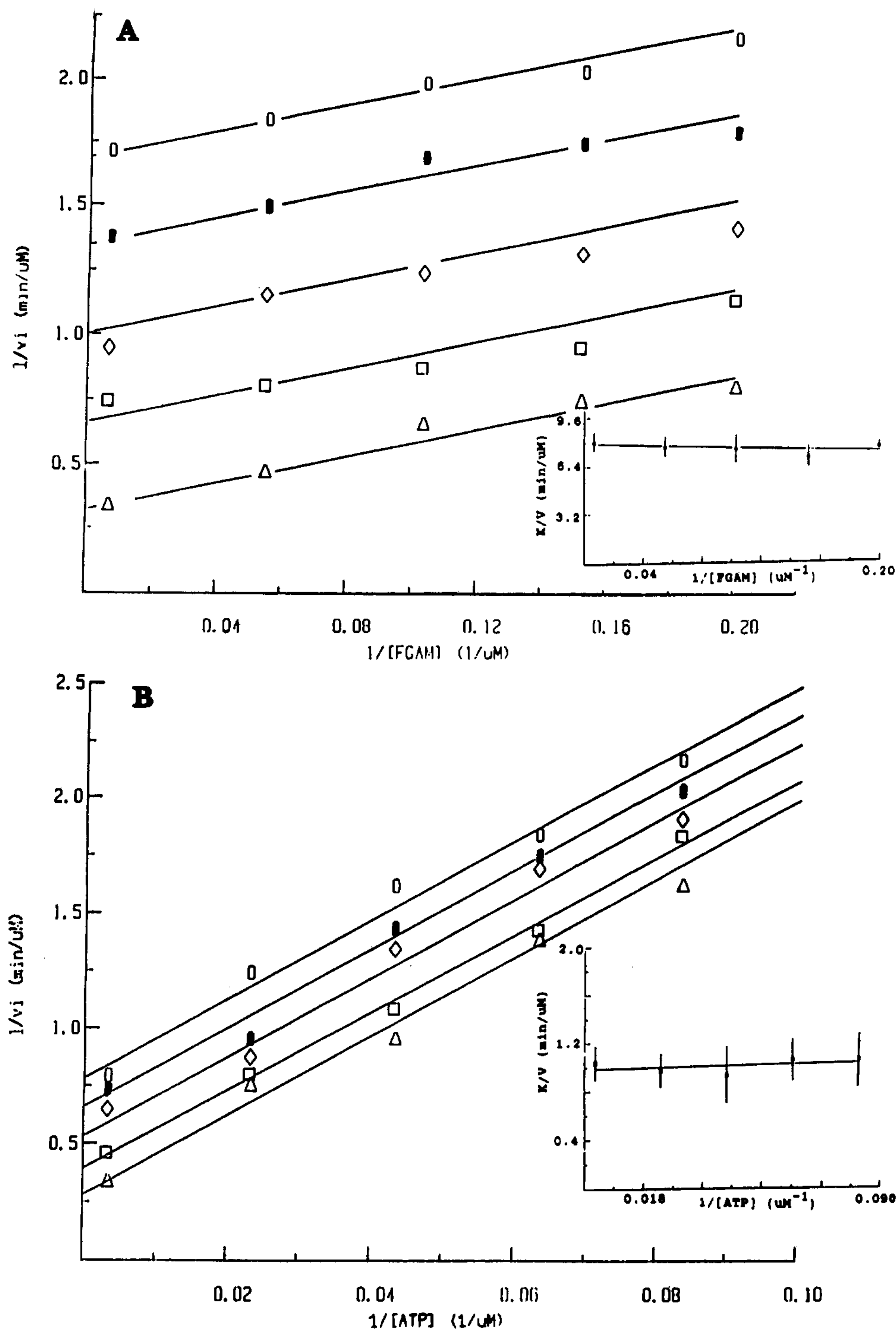


FIGURE 6: (A) Lineweaver–Burk plot of initial velocity data for AIR synthetase as a function of [β-FGAM]. ATP concentration was 5.0 (○), 6.6 (●), 9.7 (◇), 18 (□), and 300 μM (△). The straight lines were generated by using Cleland's program for parallel initial velocity patterns. The inset shows replots of the slope data. (B) Lineweaver–Burk plot of initial velocity data for AIR synthetase as a function of [ATP]. [β-FGAM] was 12 (○), 16 (●), 23 (◇), 43 (□), and 300 μM (△). The straight lines were generated as in Figure 6A. The inset shows the replot of the slope data.

Table II: Inhibition Patterns and Constants Obtained for Product Inhibition of the Reaction Catalyzed by Aminoimidazole Ribonucleotide Synthetase

variable substrate	constant substrate	inhibitor	inhibition type[a]	K_{ii} (mM)	K_{is} (mM)
ATP	FGAM (20 μM)	ADP	C		0.0169 ± 0.0019
FGAM	ATP (50 μM)	ADP	UC	0.0088 ± 0.0005	
ATP	FGAM (25 μM)	AIR	NC	2.03 ± 0.50	1.23 ± 0.36
FGAM	ATP (61 μM)	AIR	NC	1.230 ± 0.33	0.447 ● 0.026
ATP	FGAM (30 μM)	P_i	NC	338 ± 61	208 ± 58
FGAM	ATP (60 μM)	P_i	NC	185 ± 25	104 ± 12

[a] Abbreviations: C, competitive; UC, uncompetitive; NC, noncompetitive.

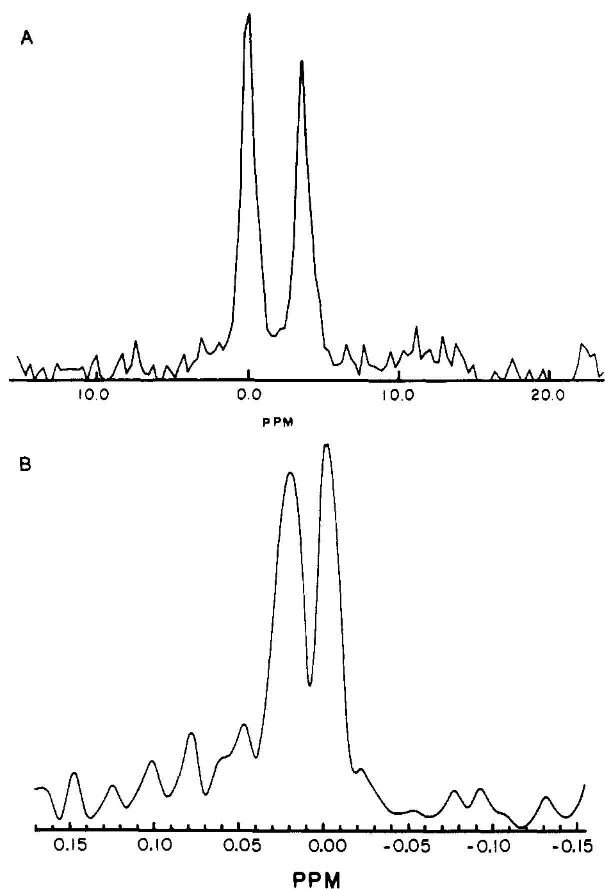


FIGURE 7: (A) Proton-decoupled 125-MHz ^{13}C NMR spectrum of [^{18}O,^{16}O,^{13}C]FGAR showing the extent of ^{18}O incorporation into the formyl position. (B) 80-MHz ^{31}P NMR spectrum of P_i showing the extent of ^{18}O incorporation into the P_i isolated from the incubation of [^{16}O,^{18}O,^{13}C]FGAM, ATP, and AIR synthetase. The [^{16}O,^{18}O,^{13}C]FGAM was prepared biosynthetically from [^{16}O,^{18}O,^{13}C]FGAR.

Procedures). The amount of ^{18}O present in this mixture was determined by examining the ^{18}O perturbation on the chemical shift of the ^{13}C resonance (Figure 7A). This labeled FGAR was then converted to β-[^{13}C,^{18}O]FGAM/[^{13}C,^{16}O]FGAM (1:1) by using FGAM synthetase, and isolated by DEAE-Sephadex A-25 chromatography which removed the P_i produced during this reaction. The labeled FGAM was then incubated with 0.8 unit of AIR synthetase in an NMR tube and its conversion to products monitored by ^{31}P NMR spectroscopy. The P_i from the reaction mixture was isolated by anion-exchange chromatography and the ^{31}P NMR spectrum recorded (Figure 7B). A 1:1 mixture of [^{18}O]- and [^{16}O]P_i was observed, indicating that the oxygen of the amide moiety of FGAM is transferred stoichiometrically to the P_i derived from ATP hydrolysis (eq 1).

Discussion

Early complementation studies using mutants of yeast deficient in purine biosynthesis in *Schizosaccharomyces pombe* (Fluri et al., 1976) and *Saccharomyces cerevisiae* (Gross & Woods, 1971) suggested that GAR synthetase and AIR synthetase activities might reside on a single bifunctional polypeptide. In fact, biochemical studies by Fluri et al. in 1976 indicated that these two activities copurified ≈100-fold from *S. pombe* and suggested that both activities were confined to a polypeptide of M_r 40 000. Interestingly, GAR synthetase was active both as a monomer (M_r 40 000) and in multimeric forms (M_r 160 000–240 000), while AIR synthetase activity could only be associated with the multimeric forms.

More recently, studies in Chinese hamster ovary (CHO) cells by Patterson et al. (1981) demonstrated coordinate regulation between the gene coding for glycinamide ribonucleotide synthetase (Ade$^-$C mutants) and the gene coding for aminoimidazole ribonucleotide synthetase (Ade$^-$G mutants). Both genes were assigned to human chromosome 21. Patterson et al. (1981) suggested a number of biochemical possibilities to account for these observations, one of them being that the two activities could be physically associated. However, similar studies by Jones et al. (1981) also assigned the GAR transformylase gene to chromosome 14 in CHO cells.

Recent studies by Caperelli et al. (1980) and Young et al. (1984) reported isolation and purification of GAR transformylase from chicken liver which was found to have a molecular weight of 110 000. These studies in conjunction with the intriguing report of Henikoff et al. (1983), that GAR transformylase was the C-terminal end of a multifunctional protein in *Drosophila*, and our recent observation that AIR synthetase had a molecular weight of 110 000 suggested that the polypeptide activity that we isolated might in fact be identical with that isolated by Young et al. A recent communication substantiated this hypothesis and allowed us to conclude that GAR synthetase, AIR synthetase, and GAR transformylase all reside on a single polypeptide of M_r 110 000 (Daubner et al., 1985). The results reported in Table I and Figures 1 and 2 indicate that the three activities (GAR synthetase, GAR transformylase, and AIR synthetase) copurify through four chromatographic steps to produce a single polypeptide of M_r 110 000, which is 90–95% pure based on SDS gels (Figure 3). While the isolation reported herein using an N-6-alkylated ATP affinity column represents substantial improvement over the early isolation of GAR transformylase reported by Caperelli et al. (1980), it is not as efficient as the more recent use of a 10-formyl-5,8-dideazafolate affinity column to isolate GAR transformylase. This procedure reported by Young et al. (1984) requires only 8 h while our procedure requires 4 days. However, the process of Young et al. requires a lengthy and difficult nine-step synthesis of the dideazafolate affinity column, which is not required in our more conventional isolation procedure. The overall yields of the two procedures are comparable, 44% and 27%, respectively.

Efforts in the present report were directed at defining the physical and catalytic properties of AIR synthetase. A number of standard methods were used to determine the apparent native molecular weight of AIR synthetase. Sephadex G-200 chromatography in 100 mM potassium phosphate (pH 7.4) and 5 mM β-mercaptoethanol in the presence or absence of the cryoprotectant 10% glycerol gave an apparent molecular weight of AIR synthetase of 330 000 when compared with a series of standards chromatographed under similar conditions. In contrast, if the potassium phosphate was replaced by 50 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol, the protein did not elute from the column. These results suggest that an unusual interaction may be occurring between the protein and the backbone of the column. Sucrose density gradient ultracentrifugation studies under conditions of similar protein concentration and buffers (phosphate or Tris-HCl) to those described for the Sephadex chromatography gave an apparent molecular weight of 133 000 on the basis of concomitant sedimentation studies with proteins of known molecular weight. This result is similar to the value of 140 000 daltons for GAR transformylase reported by Young et al. (1984) using 50 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol. In addition, SDS gel electrophoresis studies indicate a subunit molecular weight of 110 000. Hence, these

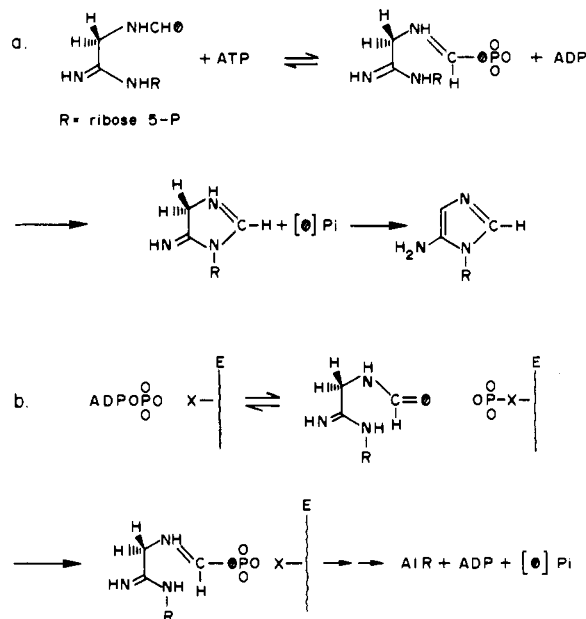
results indicate that a monomeric and a higher molecular weight aggregate can support AIR synthetase activities. These results are similar to those of Fluri et al. (1976), who investigated the molecular weight of the partially purified preparation of AIR synthetase and GAR synthetase from yeast. The real subunit stoichiometry in vivo remains to be established. However, it is interesting to speculate that the presence of other proteins involved in purine biosynthesis (for example, phosphoribosylamine synthetase, FGAM synthetase, AICAR transformylase, trifunctional protein, serine transhydroxymethylase, etc.) might alter protein conformation such that GAR transformylase, GAR synthetase, and AIR synthetase are active in monomeric or multimeric forms. Studies using analytical ultracentrifugation methodology in the presence and absence of other enzymes involved in purine biosynthesis are presently being undertaken to resolve the question of subunit stoichiometry in the native form of the protein.

The kinetic properties of AIR synthetase have also been investigated. pH studies using a wide variety of buffers at various concentrations indicate that the AIR synthetase activity has a broad pH optimum ranging from 7.5 to 8.1 with maximal activity at pH 7.7. Moreover, AIR synthetase has an absolute requirement for K^+. Na^+ cannot replace K^+; that is, in the presence of 100 mM Na^+, AIR synthetase remains inactive, and furthermore, it does not compete effectively to prevent K^+ activation (data not shown). NH_4^+ (150 mM), on the other hand, stimulates AIR synthetase activity to values identical with that observed with the same concentration of K^+. Mg^{2+} is also required for AIR synthetase activity in concentrations greater than that required to produce MgATP which is the substrate for the reaction. Initial velocity patterns and product inhibition patterns (Figure 6, Table II) were therefore determined at 150 mM K^+ and 20 mM Mg^{2+} (pH 7.7). These preliminary investigations are consistent with ATP binding first to the enzyme, followed by an irreversible step which occurs prior to the binding of the second substrate FGAM. Interpretation of the kinetic results, however, is complicated because this trifunctional protein contains an additional ATP/ADP binding site on the GAR synthetase domain. The spatial relationship of the AIR synthetase and GAR synthetase domains is presently unknown. From studies on a proteolytic fragment of GAR synthetase,[4,5] the K_m value for MgADP is 16 μM, in the same range as the K_{is} values for MgADP reported in Table II. Thus, MgADP could be acting as either a product inhibitor or a dead-end inhibitor. In the latter case, the ADP could prevent formation of the active enzyme form with which FGAM combines, hence accounting for the uncompetitive product inhibition (Table II).

Furthermore, we have recently isolated AIR synthetase from *E. coli* which is a monofunctional dimer (71 700 daltons). Preliminary initial velocity kinetic investigations using ATP and FGAM with this protein indicate intersecting patterns, rather than the parallel pattern observed with the chicken liver protein. These results tend to reinforce the point that interpretation of the kinetic studies from the chicken liver protein might be premature given the unknown relationship between the AIR synthetase and GAR synthetase binding domains.

Characterization of FGAM and AIR. No chemical methods are presently available to synthesize β-FGAM or AIR. We therefore modified the biosynthetic procedures reported by Lukens and Flaks (1963) and prepared these compounds and characterized them by ^{1}H and in the latter case ^{1}H and ^{13}C NMR spectoscropy (Figures 4 and 5). We are presently investigating the chemical stability of these compounds and the nature of breakdown products produced.

Scheme I



Mechanistic Studies. Conversion of FGAM to AIR involves activation of the formyl oxygen of FGAM by ATP toward nucleophilic displacement by the 1′-N. This activation may occur by any number of mechanisms as indicated in Scheme I. All of these mechanisms predict the oxygen of the formyl group of FGAM will be transferred to P_i. This transfer was shown to occur by preparation of a 1:1 mixture of [^{18}O,^{13}C]FGAM/[^{16}O,^{13}C]FGAM. Incubation of this material with AIR synthetase produced a 1:1 mixture of [^{18}O]P_i[^{16}O]P_i as shown by the ^{18}O perturbation of the ^{31}P resonance of P_i (Figure 7B).

To distinguish between a mechanism such as the one shown in Scheme Ib, which indicates a covalent phosphorylated enzyme intermediate, and the one shown in Scheme Ia, two methods are available: (1) phosphorus stereochemical studies and (2) ATP/ADP exchange studies. In the former case, using chiral [^{18}O]ATPγS, the observed stereochemistry of the chiral thiophosphate produced would be retention of configuration, whereas in Scheme Ia, the observed stereochemistry would be inversion of configuration. We have shown that ADPγS is an alternate substrate for AIR synthetase, producing thiophosphate at $^1/_{100}$th the normal rate of turnover with a K_m = 87 ± 29 μM. Stereochemical studies are in progress which should allow us to make this mechanistic distinction. In addition, preliminary ATP/[^{14}C]ADP exchange studies indicate that exchange does occur, but at $^1/_{20}$th the rate of AIR production. While the observed ATP/ADP exchange and the lack of ATP/P_i exchange are consistent with production of a phosphorylated enzyme intermediate, the slowness of the exchange rates is cause for concern as to its relevance to the normal catalytic pathway.

Summary

AIR synthetase has been purified to homogeneity via four successive chromoatographic procedures. Interestingly, both GAR transformylase and GAR synthetase copurify, with AIR synthetase maintaining a constant ratio of specific activities throughout the isolation. Thus far, of the 10 reactions involved

[4] M. Murray, F. Schendel, Y.-S. Cheng, and J. Stubbe, unpublished results.

[5] GAR synthetase has been isolated by an independent procedure[4] and shown to be a monomer of M_r 54 000. The turnover number is identical with that observed for the 110-kilodalton protein reported in this paper.

in production of purines, that is, conversion of phosphoribosyl pyrophosphate to IMP, 4 multifunctional proteins have been identified: a bifunctional enzyme that exhibits aminoimidazolecarboxamide ribonucleotide transformylase and IMP cyclohydrolase activities (Benkovic, 1984), a bifunctional protein which possesses aminoimidazole ribonucleotide carboxylase and aminoimidazolesuccinocarboxamide ribonucleotide synthetase activities (Patey & Shaw, 1973), a bifunctional protein adenylosuccinate lyase and 5-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole lyase (Woodward, 1978), and the trifunctional protein containing GAR synthetase, GAR transformylase, and AIR synthetase (Daubner et al., 1985; present study). Furthermore, similar studies to the genetic experiments of Patterson et al. (1981) which suggested that AIR synthetase might be a bifunctional protein also suggest that phosphoribosylamine synthetase and FGAM synthetase might be a bifunctional protein, or share a common glutamine domain. Evidence thus far indicates that these are the only two monofunctional proteins in the pathway. Studies are presently in progress in our laboratory using antibodies prepared to FGAM synthetase to test this possibility. Our initial hypothesis for considering the advantage of bifunctional protein structure was based on the reported instability of purine biosynthetic intermediates such as phosphoribosylamine, FGAM, and AIR. The idea of channeling of unstable intermediates is exemplified by the elegant studies of Benkovic and co-workers with enzymes involved in the formylation processes of purine biosynthesis, namely, GAR transformylase, AICAR transformylase, trifunctional protein, and serine transhydroxymethylase which form a noncovalent complex through several stages of purification (Benkovic, 1984). The nature of the interaction of AIR synthetase, GAR synthetase, and GAR transformylase with other purine biosynthetic enzymes and their regulation await more detailed biochemical investigations.

ACKNOWLEDGMENTS

We thank John Hansen for recording the 125-MHz ^{13}C NMR spectrum in the Chemistry Department at the University of California, Berkeley.

Registry No. P_i, 14265-44-2; β-FGAM, 6157-85-3; ADP, 58-64-0; AIR, 25635-88-5; MgATP, 1476-84-2; AIR synthetase, 9023-53-4; GAR synthetase, 9032-01-3; GAR transformylase, 9032-02-4.

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