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Substrate Specificity of Formylglycinamidine Synthetase†

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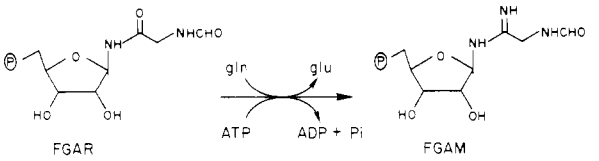
ABSTRACT: Formylglycinamidine ribonucleotide (FGAM) synthetase, which catalyzes the conversion of formylglycinamide ribonucleotide (FGAR), glutamine, and ATP to FGAM, ADP, glutamate, and P_i, has been purified to homogeneity (sp act. 0.20 μmol min^{-1} mg^{-1}) from chicken liver by an alternative procedure to that of Buchanan et al. [Buchanan, J. M., Ohnoki, S., & Hong, B. S. (1978) *Methods Enzymol. 51*, 193–201] (sp act. 0.12 μmol min^{-1} mg^{-1}). A variety of new analogues of formylglycinamide ribonucleotide have been prepared in which the formylglycinamide arm (R = CH_2NHCHO) has been replaced by R = CH_3, CH_2OH, CH_2Cl, CH_2NH_3, $CH_2NHCOCH_3$, $CH_2NHCOCH_2Cl$, $CH_2NHCO_2CH_2Ph$, and L-$CHCH_3NHCHO$. These compounds have been characterized by ^1H and ^{13}C NMR spectroscopy. With compounds R = CH_3, CH_2OH, and $CH_2NHCOCH_3$ and ATP, in the presence or absence of glutamine, FGAM synthetase catalyzes the production of P_i at 4.5, 48, and 20%, respectively, the rate of production of P_i from formylglycinamide ribonucleotide. Only R = $CH_2NHCOCH_3$ causes glutaminase activity as well as ATPase activity and has been shown to be converted to the amidine analogue. Both FGAR (R = CH_2NHCHO) and the FGAR analogue (R = $CH_2NHCHOCH_3$) in the presence of ATP and FGAM synthetase and in the absence of glutamine form a complex isolable by Sephadex G-50 chromatography. FGAM synthetase is thus highly specific for its formylglycine side chain. [^{18}O]-β-FGAR was prepared biosynthetically, and FGAM synthetase was shown by ^{31}P NMR spectroscopy to catalyze the transfer of amide ^{18}O to inorganic phosphate.

Formylglycinamidine ribonucleotide (FGAM)[1] synthetase catalyzes the fourth step in the purine biosynthetic pathway conversion of formylglycinamide ribonucleotide (FGAR), glutamine, and ATP to FGAM, P_i, and ADP (eq 1). This



(1)

enzyme has been previously purified 300-fold to homogeneity from chicken liver and found to consist of a single polypeptide chain of M_r 133 000 (Mizobuchi & Buchanan, 1968a; Buchanan et al., 1978). Many detailed mechanistic studies have been undertaken by Buchanan and co-workers and have been recently reviewed (Buchanan, 1982). FGAM synthetase has also been purified 56-fold from Erlich ascites tumor cells by Chu and Henderson (1972). Mechanistic studies on this partially purified protein indicate considerable differences between it and the liver enzyme.

Elegant studies by Buchanan and co-workers have elucidated the role of glutamine in this enzyme-catalyzed amidine production. Their studies with [^{14}C]glutamine indicated that in the absence of other substrates the enzyme forms a 1:1 complex with glutamine ($t_{1/2}$ = 125 min). Futher studies indicate that a covalent thio ester of glutamine is formed enzymatically with liberation of "NH_3". This "NH_3" is the putative nucleophile in the FGAR → FGAM conversion and remains associated with a specific locus in the active site. Hydrolysis of the enzyme glutamyl moiety in the absence of other substrates is 0.5% the rate of turnover for the overall reaction (Mizobuchi & Buchanan, 1968b).

Intriguingly, a stable complex is also formed between ATP, FGAR, and enzyme with a stoichiometry of 0.7 mol of substrate/mol of enzyme in the absence of glutamine ($t_{1/2}$ = 62 min at 0 °C). Furthermore, there is evidence that the terminal phosphoanhydride bond of ATP is cleaved to ADP and a phosphate moiety (perhaps E∼PO_3) during this complex formation (Mizobuchi et al., 1968).

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[1] Abbreviations: FGAM, formylglycinamidine ribonucleotide; FGAR, formylglycinamide ribonucleotide; GAR, glycinamide ribonucleotide; AIR, aminoimidazole ribonucleotide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PRPP, 5-phosphoribosyl pyrophosphate; PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FID, free induction decay; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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While a great deal is now known about the glutamine binding site of FGAM synthetase, little is known about the nature of the FGAR and ATP binding sites and the mechanism of amide to amidine conversion. This in part is due to the synthetic difficulties in preparation of FGAR derivatives, as exemplified by the fact that in all of Buchanan's early work and many recent papers investigating purine biosynthesis FGAR is prepared biosynthetically.

In this paper, an alternate improved isolation procedure of FGAM synthetase from chicken liver is reported. A variety of FGAR analogues have been prepared chemically and characterized by ^{13}C and ^{1}H NMR spectroscopy. These analogues have been incubated with FGAM synthetase, glutamine, and ATP and analyzed for their ability to cause conversion of glutamine to glutamate and ATP to ADP and P_i, as well as amide to amidine. [^{18}O]-β-FGAR was prepared biosynthetically and the fate of ^{18}O determined by ^{31}P NMR spectroscopy. These studies have better defined the FGAR binding site of FGAM synthetase.

Materials and Methods

GAR was prepared by the procedure of Chettur and Benkovic (1977). 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, acetate, chloroacetate, hydroxyacetate, formylglycine, L-formylalanine, and *tert*-butyldimethylsilyl chloride were purchased from Aldrich. *N*-Acetylglycine, *N*-(chloroacetyl)glycine, DEAE-Sephadex A-50, CM-Sephadex C-50, Sephadex G-25, Sephacryl S-200, 3-acetylpyridine adenine dinucleotide, glutamate dehydrogenase (50 μmol min^{-1} mg^{-1}), phosphoenolpyruvic acid, folinic acid, PRPP, pyruvate kinase (355 μmol min^{-1} mg^{-1}), BSA, and ATP were obtained from Sigma Chemical Co. Azaserine was obtained from P-L Biochemicals. [1-^{13}C]Glycine (>99% ^{13}C) was obtained from MSD isotopes. $H_2{}^{18}O$ (95.1% ^{18}O) was obtained from Monsanto Research Corp. AIR synthetase was isolated from *Escherichia coli* clone pJS-24, provided to us by John Smith from the Louisiana State University Medical School, Shreveport, LA. It had a specific activity of 4 μmol min^{-1} mg^{-1}. [^{32}P]Orthophosphoric acid, carrier free, and [1-^{14}C]-glycine (49.3 mCi/mmol) were obtained from New England Nuclear. [γ-^{32}P]ATP was prepared by the procedure of Selman and Selman-Reimer (1981). [^{18}O]Glycine was prepared by the procedure of Mears and Sobotka (1939). Hydroxylapatite (HPT) was purchased from Bio-Rad Corp. All other reagents were of highest purity available. NMR spectra were recorded on either a Bruker 270-MHz spectrometer, a Nicolet 200-MHz broad band spectrometer, or an IBM NR-80 spectrometer. All solutions were analyzed for radioactivity in Packard Scint-A scintillation fluid and on a Packard 310 liquid scintillation counter. Protein concentrations were determined by the procedure of Lowry et al. (1951) with BSA as a standard. SDS gel electrophoresis was carried out by the method of Laemmli (1970). Orcinol assays were performed as described by Dische (1962). Phosphate assays were performed as described by Ames and Dubin (1960).

Enzyme Assays. FGAM synthetase was assayed by the procedure of Mizobuchi and Buchanan (1968a) with the following modifications. The FGAR used was a 1:1 mixture of α- and β-anomers synthesized as described below, and the final volume of the reaction mixture was 400 μL containing 50 mM Tris-HCl (pH 8.0), 2.0 mM glutamine, 2 mM α/β-FGAR, 80 mM KCl, 20 mM $MgCl_2$, 10 mM ATP, AIR synthetase (0.05 unit), and varying amounts of FGAM synthetase. The reaction was incubated at 37 °C for 10 min and quenched by the addition of 100 μL of 20% TCA in 1.33 M potassium phosphate, pH 1.4. After the protein was spun down by centrifugation, a modified Bratton–Marshall assay (Bratton & Marshall, 1939) was carried out by addition of 100 μL of 0.1% sodium nitrite. After 3 min, 100 μL of 0.5% ammonium sulfamate was added, and after an additional 1 min, 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride was added and the color that formed allowed to develop for 10 min. The pink color was read at 500 nm, and the turnover was calculated with the extinction coefficient of 24 600 M^{-1} cm^{-1} as determined by Levenberg and Buchanan (1957) and confirmed in this laboratory.[2] The coupling enzyme AIR synthetase was prepared from pigeon liver as described by Flaks and Lukens (1963) or was purified to homogeneity from an *E. coli* clone, pJS-24. One unit of enzymatic activity is defined as the amount of enzyme required to convert 1 μmol of FGAR to FGAM per minute, under standard assay conditions.

Glutaminase Assays. Glutaminase assays were carried out by the procedure of Buchanan et al. (1978). The reaction was carried out in a final volume of 300 μL containing 50 mM Tris-HCl (pH 8.0), 80 mM KCl, 20 mM $MgCl_2$, 10 mM ATP, 2 mM glutamine, 11 mM α/β-VI, and various amounts of FGAM synthetase. The reaction was incubated at 37 °C. At various time intervals, a 50-μL sample was withdrawn and added to 150 μL of water in a 1.5-mL microcentrifuge tube. The tube was placed in a boiling H_2O bath for 1 min and cooled on ice; then, 30 μL of 60 mM 3-acetylpyridine adenine dinucleotide containing 2 units of glutamate dehydrogenase was added. The reaction was incubated for 2 h at 37 °C and the diluted with 370 μL of water. The absorbance was read at 363 nm and the amount of glutamate formed calculated with 8.3 mM^{-1} cm^{-1} as the extinction coefficient. A control reaction was run under conditions identical with those described above, which contained known amounts of glutamate in place of the FGAR analogue.

ATPase Assays. The reaction was carried out in a final volume of 60 μL containing 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 80 mM KCl, 10 mM [γ-^{32}P]ATP (1×10^7 cpm/μmol), 0.01–10 mM FGAR analogue, 2 mM L-glutamine, and various amounts of FGAM synthetase. The reaction mixture without enzyme was incubated at 37 °C for 1 min, and then the reaction was started by the addition of FGAM synthetase. At various time intervals, 10 μL of the reaction mixture was withdrawn and added to 2 mL of "acid molybdate solution" (80:20 1 N perchloric acid–5% ammonium molybdate) containing 100 μL of 0.1 M potassium phosphate. Then, 2 mL of IBB (1:1 mixture of 2-methyl-2-propanol–benzene) was added; the mixture was vortexed for 15 s and centrifuged for 15 s to separate the layers (Vallejos, 1973). The inorganic phosphate produced was determined by liquid scintillation counting of 1 mL of the 2-mL top layer. The apparent K_m for the FGAR analogue was determined by varying the concentration of the analogue, measuring the initial velocity, and fitting the data to the kinetic programs of Cleland (1979).

Amidine Formation from VI (Table III). The reaction was carried out in a final volume of 500 μL containing 50 mM HEPES (pH 7.8), 20 mM $MgCl_2$, 80 mM KCl, 10 mM [γ-^{32}P]ATP (3.6×10^6 cpm/μmol), 7 mM α/β-VI, and 2 mM L-glutamine. This mixture was incubated at 37 °C for 1 min and the reaction started by the addition of 1.5 units of FGAM synthetase. After 10 min at 37 °C, 10 μL of the reaction was withdrawn and the amount of inorganic [^{32}P]phosphate produced determined as described above. The reaction mixture

[2] J. L. Schrimsher and J. Stubbe, unpublished results.

was applied to a column of DEAE-Sephadex A-25 (0.38 × 4 cm) equilibrated with 10 mM ammonium acetate, pH 5.0. The column was washed with this buffer, and 1-mL fractions were collected. Fractions 2–4 were pooled and adjusted to pH 8.0 with 50 mM sodium hydroxide, and the sample was applied to another DEAE-Sephadex A-25 (0.38 × 4 cm) column. The column was washed with 8 mL of water; then, the amidine was eluted with 200 mM ammonium acetate, pH 8.0. One-milliliter fractions were collected and assayed for pentose (Dische 1962) and phosphate (Ames & Dubin, 1960).

Purification of FGAM Synthetase. All steps were carried out at 4 °C unless otherwise stated, and all buffers contained 1 mM L-glutamine, 10% glycerol, and 5 mM β-mercaptoethanol. Chicken liver was obtained from Arcadia Fryers, Arcadia, WI, frozen in liquid nitrogen, and stored at −80 °C. The evening before an enzyme preparation, the liver, 100 g, was placed in a −20 °C freezer. The liver was homogenized in 2.5 volumes, 250 mL, of 10 mM potassium phosphate (pH 7.2) containing 1 mM phenylmethanesulfonyl fluoride in a Waring blender for 2 min at high speed. The homogenate was centrifuged at 23000*g* for 20 min. The supernatant was decanted from the pellet through two layers of cheesecloth into a beaker where it was brought to 45% saturation in ammonium sulfate by the addition of solid ammonium sulfate (277 g/L) over 20 min. The solution was then stirred for 20 min and the precipitated protein collected by centrifugation at 23000*g* for 20 min. After the supernatant was decanted, the pellet was redissolved in 200 mL of the homogenization buffer and the pH adjusted to 4.8 by the slow addition of 1 N acetic acid (10–25 mL was usually required). Once the solution had reached pH 4.8, the protein was stirred for 5 min and then centrifuged at 23000*g* for 10 min. The supernatant was collected and the pH adjusted to 7.2 by the slow addition of 1 N ammonium hydroxide. This solution was again brought to 45% saturation in ammonium sulfate by the same procedure described above. After centrifugation, the pellet was collected and redissolved in a minimum volume of homogenization buffer (35–50 mL). This solution was then applied to a Sephadex G-25 column (4.5 × 45 cm) equilibrated in the homogenization buffer. The desalted protein was collected and diluted with 2 volumes of distilled water. The protein solution was then applied to a DEAE-Sephadex A-50 column (4 × 25 cm) equilibrated with 10 mM potassium phosphate (pH 7.2). The column was washed with this buffer until the absorbance at 280 nm was less than 0.1; then, the column was washed with 10 mM potassium phosphate (pH 7.2) containing 50 mM potassium chloride until the absorbance at 280 nm was less than 0.1. The enzyme was eluted from the column by 10 mM potassium phosphate containing 100 mM potassium chloride. The fractions containing activity were pooled and concentrated in an Amicon ultrafiltration apparatus with a PM-30 membrane.

After concentration, the enzyme was dialyzed overnight against 20 mM potassium phosphate (pH 6.5). The enzyme was applied to a hydroxylapatite column (Bio-Rad, HPT) (1.5 × 7 cm) equilibrated in 20 mM potassium phosphate (pH 6.5). The enzyme did not bind under these conditions; however, most of the contaminating proteins did. The fractions that contained activity were pooled, concentrated by ultrafiltration, and stored at −80 °C. The enzyme was stable for several months if stored under these conditions.

An alternative procedure to the hydroxylapatite column was to dialyze the enzyme against 10 mM potassium citrate (pH 5.5). This solution was then applied to a CM-Sephadex C-50 (2 × 12 cm) column equilibrated in 10 mM potassium citrate (pH 5.5). The column was washed with this buffer until the absorbance at 280 nm was less than 0.1, and then the enzyme was eluted with 10 mM potassium citrate (pH 5.5) containing 80 mM potassium chloride. The fractions containing activity were pooled, concentrated, dialyzed against 20 mM potassium phosphate, pH 6.5, and stored at −80 °C.

Another procedure that could replace the hydroxylapatite or CM-Sephadex methods was to concentrate the enzyme from the A-50 column to a volume of 5 mL or less and apply it to a Sephacryl S-200 column (2.5 × 120 cm) equilibrated in 20 mM potassium phosphate, pH 6.5. The column was run at a flow rate of 6 mL/h, and the fractions that contained activity were pooled, concentrated, and stored at −80 °C.

General Synthetic Approach to FGAR Analogues. The general procedure involves extensive modifications of an earlier procedure of Chu and Henderson (1970). 2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl azide (**1**) was prepared by a modification of the procedure of Camarasa et al. (1980). The amount of $SnCl_4$ was reduced to $^1/_{10}$ that originally reported, and the overall yield on the reaction subsequent to recrystallization was 95%. To 3.0 g (6 mmol) of **1** in 120 mL of anhydrous acetone 150 mg of PtO_2 was added, and the mixture was hydrogenated at 10 psi for 45 min at room temperature. After removal of H_2 gas by repeated evacuation and N_2 flushing, 2 g of $MgSO_4$ was added to the solution, which was then filtered through Celite into a 250-mL round-bottom flask containing 11 mmol of the appropriate acid in 10 mL of dry acetone. Dicyclohexylcarbodiimide was added (1.8 g, 8.7 mmol), and the mixture was allowed to stir at room temperature for 3 h. At that time, the dicyclohexylurea was removed by suction filtration, and the filtrate was concentrated in vacuo. After removal of approximately half of the acetone, any dicyclohexylurea that precipitated was removed by filtration. The remainder of the acetone was removed in vacuo, and the syrupy residue was dissolved in a minimal volume of $CHCl_3$ (from which the ethanol stabilizer had not been removed) and chromatographed on a column (2.5 × 50 cm, 110 g) of silica gel (Kieselgel 60) (70–230 mesh) with 1% CH_3OH in $CHCl_3$ as an eluant. This procedure allows separation of the α and β isomers; however, in general the mixture of anomers was utilized in all subsequent steps. Typical yields of blocked nucleosides ranged from 60 to 80%.

Removal of the blocking groups was effected by dissolving 1 mmol of the blocked nucleoside in 10 mL of CH_3OH in a 25-mL round-bottom flask fitted with a stir bar and drying tube. Sodium methoxide (0.5 mmol) in 1 mL of CH_3OH was added, and the reaction was followed by thin-layer chromatography on silica gel with $CHCl_3$ as developer. When all of the benzoyl groups had been converted to methyl benzoate, typically 15–30 min, the reaction was neutralized by addition of Dowex 50W-X8 (H^+ form). The Dowex was then removed by suction filtration, and the CH_3OH in the filtrate was removed in vacuo. The residue was dissolved in 25 mL of H_2O and extracted with 2 × 25-mL portions of ethyl ether. The H_2O layer was then concentrated to dryness in vacuo. In general, the yields on the deblocking step were >90%.

Phosphorylation of the nucleoside was accomplished with the general procedure of Yoshikawa et al. (1967). The α/β mixture of the nucleoside (180 μmol) was placed in a 10-mL pear-shaped flask and dried over P_2O_5 in vacuo overnight. To the flask was then added 2 mL of freshly distilled $PO(OEt)_3$. The reaction mixture was cooled to 0 °C, and 0.2 mL (10 equiv, 2 mmol) of $POCl_3$ (freshly distilled) was added. The reaction was stirred for 1.5 h at 0 °C. In general, the ether precipitation method that worked in the case of FGAR was

unsuccessful with the majority of the analogues. The general workup, therefore, involved addition of 2 mL of cold H_2O followed by rapid titration of the solution to pH 7.5 with 1 N NaOH. NaOH was added periodically over the period of 1 h to maintain the pH at 7.5, at which time the excess $POCl_3$ had been hydrolyzed. The solution was then diluted to 100 mL with ice–H_2O and applied to a (1.8 × 30 cm) DEAE-Sephadex A-25 column. The product was eluted with a 1-L linear gradient of triethylammonium bicarbonate (0–400 mM) (pH 7.8). The inorganic phosphate peak was detected by phosphate assays (Ames & Dubin, 1960). The organic and inorganic phosphates were separated by standard barium precipitation procedures (Cardini & Leloir, 1957). The barium salt of the nucleotide was exchanged for sodium on Dowex 50W-X8 (Na^+ form), and the ^1H and ^{13}C NMR's were recorded (Tables III and IV). In general, the yields ranged from 20 to 30%. In all cases, the ratio of α- and β-isomers was determined by integration of the C-1′ anomeric protons. The concentration of the various analogues was determined by comparing the phosphate concentration (Ames & Dubin, 1960) with the pentose concentration (Dische, 1962). The concentration of the β anomer used in the determination of K_m values was calculated from the NMR, phosphate, and orcinol assay data.

Preparation of II (Table III). Preparation of II required a different protocol from that described above, due to the reactive primary hydroxyl moiety. To 1 g (2.0 mmol) of *N*-(hydroxyacetyl)-2,3,5-tri-*O*-benzoyl-$\alpha\beta$-D-ribofuranosylamine in 1.5 mL of DMF (distilled from CaH_2) was added 332 mg (4.8 mmol) of imidazole and 340 mg (2.3 mmol) of *tert*-butyldimethylsilyl chloride. The reaction was stirred for 24 h at room temperature. The DMF was removed in vacuo and the reaction product dissolved in 50 mL of CH_2Cl_2 and extracted 3 × 10 mL with H_2O. The CH_2Cl_2 layer was dried over $MgSO_4$ and filtered and the solvent removed in vacuo: NMR ($CDCl_3$) δ 0.09 (s, 6 H), 0.15 (s, 6 H), 0.89, (s, 9 H), 0.97 (s, 9 H), 4.13 (s, 2 H), 4.16 (s, 2 H), 4.6–4.8 (m, 6 H), 5.5–6.2 (m, 6 H), 7.2–7.8 (m, 30 H). The benzoyl blocking groups were removed, and the nucleoside was phosphorylated as described above. Workup of the phosphorylated nucleoside also removes the *tert*-butyldimethylsilyl blocking group.

Isolation of FGAR Biosynthetic Enzymes. The purine biosynthetic enzymes were partially purified by a modification of the procedure of Rowe and Wyngaarden (1968). Pigeon liver (15 g, fresh or stored at −80 °C after being frozen in liquid nitrogen) was homogenized in 2 volumes of 50 mM Tris-HCl (pH 8.0), 250 mM sucrose, and 1 mM $MgCl_2$ and centrifuged for 30 min at 27000*g*. The pH of the supernatant was then adjusted to 5.1 with 1 N acetic acid and allowed to stand for 20 min at 4 °C. After centrifugation at 27000*g* for 10 min, the pH of the supernatant was readjusted to 6.0 with 1 N ammonium hydroxide. The solution was then brought to 35% saturation in ammonium sulfate by the addition of solid ammonium sulfate (209 g/L) and allowed to stir for 2 h at 4 °C. After centrifugation at 27000*g*, the supernatant was dialyzed overnight against 50 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$ to give 25 mL, 40 mg/mL in protein.

Synthesis of [^{13}C,^{18}O/^{16}O]FGAR. To the biosynthetic reaction mixture containing 30 μmol of PRPP, 80 μmol of L-glutamine, 10 μmol of [^{13}C,^{18}O]glycine (^{18}O content >95% by ^{13}C NMR), 10 μmol of [^{13}C,^{16}O]glycine (Hyman, 1957), 1.0 μCi of [1-^{14}C]glycine (sp act. 49.3 mCi/mmol), 100 μmol of ATP, 100 μmol of PEP, 400 μmol of $MgCl_2$, 30 μmol of folinic acid, 2 μmol of azaserine, and 5 units of pyruvate kinase in 10 mL of 50 mM Tris-HCl (pH 8.0) was added 10 mL of the purine biosynthetic enzymes, 400 mg of protein. The reaction mixture was incubated at 37 °C for 90 min and then quenched with 1 mL of 60% TCA. The precipitated protein was removed by centrifugation and the pellet washed 3 times with 5 mL of 5% TCA. The combined supernatants were diluted to 300 mL with water, adjusted to pH 8.0 with 3 N KOH, and then applied to a DEAE-Sephadex A-25 column (2.5 × 18 cm). The column was washed with 2 volumes of water, and the FGAR was eluted with an 800-mL linear gradient from 0 to 400 mM triethylammonium bicarbonate. The fractions containing FGAR eluted at 200 mM triethylammonium bicarbonate and were located by scintillation counting, pooled, and evaporated to dryness in vacuo. The FGAR was redissolved in 10 mL of water, the pH adjusted to 8.5 with 3 N KOH, and 200 μmol of barium bromide added. The precipitated barium phosphate that formed was removed by centrifugation and the pH of the supernatant readjusted to 8.5. Five volumes of cold absolute ethanol was added and the solution placed at −20 °C for 4 h. The precipitate that formed was collected, dried over P_2O_5 in vacuo, and dissolved in water, and the barium salt was exchanged for sodium by passage through Dowex 50W-X8 (Na^+ form): yield 10 μmol; proton NMR (D_2O, pD 6.0) δ 3.69 (d, 2 H, $J_{^{13}CCH}$ = 4.8 Hz, CH_2 of formylglycine), 5.14, (dd, 1 H, $J_{^{13}CNCH}$ = 2.0 Hz, $J_{1'-2'}$ = 5.0 Hz, 1′), 7.86, (s, 1 H), formyl proton).

^{18}O Transfer Experiment. To a 10-mm NMR tube containing 60 μmol of ATP, 60 μmol of L-glutamine, 240 μmol of KCl, 120 μmol of $MgCl_2$, and 100 μmol of Tris-HCl, pH 8.0, in a final volume of 2 mL (20% D_2O) was added 0.5 unit of FGAM synthetase (0.20 unit/mg). The ^{31}P NMR spectrum was taken to 80 MHz, and then 6.5 μmol of [^{18}O]FGAR was added and the tube incubated at 37 °C for 30 min. When the reaction was complete, as determined by ^{31}P NMR, the reaction mixture was diluted to 25 mL with water and passed through an Amicon ultrafiltration apparatus with a PM-30 membrane. The filtrate was collected and applied to a DEAE-Sephadex A-25 column (0.5 × 4 cm). The column was washed with 2 volumes of water, and the inorganic phosphate was eluted with 200 mM triethylammonium bicarbonate (pH 7.8). The fractions containing the inorganic phosphate were pooled, concentrated to dryness in vacuo, and subjected to ^{31}P NMR (80-MHz) analysis. The ratio of ^{16}O:^{18}O-labeled P_i produced was determined by weighing the appropriate ^{31}P peaks and found to be 51:49, respectively. A sweep width (±1000 Hz), quadrature phase detection, a 90° pulse angle, and a 16K data block were used. The acquisition time was 3.4 s, and the pulse delay was 2 s. To enhance resolution, exponential multiplication with a line-broadening factor of 0.1 Hz was applied to the FID before Fourier transformation.

Nature of ATPase by I. The reaction was carried out in a 5-mm NMR tube with a final volume of 1.0 mL (30% D_2O) containing 10 mM Tris-HCl (pH 8.0), 80 mM KCl, 20 mM $MgCl_2$, 60 mM ATP, 40 mM α/β-I, 8 units of FGAM synthetase, and 500 μL of $H_2{}^{18}O$ (^{18}O content 45.1%). The reaction was incubated at 37 °C and followed by ^{31}P NMR. After the reaction had produced between 5 and 10 μmol of inorganic phosphate, the enzyme was removed by ultrafiltration through a PM-30 membrane with an Amicon ultrafiltration apparatus. The inorganic phosphate was isolated by DEAE-Sephadex chromatography and subjected to ^{31}P NMR analysis as described above.

ATP, FGAM Synthetase, FGAR, or VI Complex. The incubations were carried out in a total volume of 400 μL containing 25 mM potassium phosphate (pH 6.5), 20 mM $MgCl_2$, 80 mM KCl, 20 mM [γ-^{32}P]ATP (2.8 × 10^6 cpm/

Table I: Isolation of FGAM Synthetase

method	total protein (mg)	protein (mg/mL)	total act. (units)	sp act. (units/mg of protein)
crude[a]	9611	33	1.8	0.002
$(NH_4)_2SO_4$	3200	16	3.2	0.001
acid	1320	6.5	6.6	0.005
$(NH_4)_2SO_4$	840	4.0	8.4	0.01
DEAE-Sephadex A-50	132	0.4	6.6	0.05
hydroxylapatite	18.5	0.29	3.7	0.20
DEAE-Sephadex A-50[b]	180	0.45	9.7	0.05
CM Sephadex C-50	47	0.5	4.8	0.10
A-50[c]	167	0.54	7.5	0.05
Sephacryl S-200	24	0.95	4.0	0.16

[a] A total of 75 g of chicken liver. [b] A different preparation starting with 100 g of chicken liver. [c] A third preparation starting with 100 g of chicken liver.

μmol), 0.05 unit of FGAM synthetase, and 9 mM of either α/β-FGAR or FGAR analogue. The reactions were incubated at 37 °C for 5 min and then applied to a Sephadex G-50 column (0.75 × 21 cm) equilibrated in 100 mM potassium phosphate, pH 6.5. Fractions of 1 mL were collected, the absorbance at 280 nm was recorded, and 500 μL of each fraction was analyzed for ^{32}P in a liquid scintillation counter. The molar ratio of [γ-^{32}P]ATP bound to the enzyme was calculated with 196 000 M^{-1} cm^{-1} as the extinction coefficient for the enzyme at 280 nm (Mizobuchi et al., 1968).

Results and Discussion

FGAM Synthetase Purification. The most recent purification of FGAM synthetase (Ohnoki et al., 1977; Buchanan et al., 1968), a modification of the original procedure of Mizobuchi and Buchanan (1968a), reports isolation of 57 mg of protein of specific activity 0.12 μmol min^{-1} mg^{-1} in 24% overall yield, starting with 475 g of chicken liver.[3] We encountered a number of difficulties in attempting to reproduce this isolation and therefore developed an alternative procedure as indicated in Table I. This procedure gives 18.5 mg of protein of specific activity 0.20 μmol min^{-1} mg^{-1} in 43% overall yield starting with 74 g of chicken liver. The enzyme appears to be ≈90% pure on the basis of SDS gel electrophoresis (Figure 1) and has a M_r 133 000, as reported earlier. Antibodies prepared to homogeneous FGAM synthetase and used in Western blot studies indicate that the 5–10% contaminating proteins are cross-reactive with the antibody and, hence, most probably are proteolytic fragments of FGAM synthetase. It should be noted that Buchanan's specific activities, as well as our own, are based on the conversion of the FGAM produced during this reaction to aminoimidazole ribonucleotide (AIR) with AIR synthetase. We have recently purified this enzyme to homogeneity from *E. coli* and chicken liver (Schrimsher et al., 1986).[2] Hence, our coupling enzyme does not possess the problems that might be encountered with the ethanol fraction of pigeon liver possessing AIR synthetase used by earlier workers (Flaks & Lukens, 1963).

The isolation procedure described in Table I for purification of FGAM synthetase represents the most reproducible and simplest methodology developed to date. While we purified this protein to apparent homogeneity by a variety of other procedures, the reproducibility depended on the time of year we obtained the chicken livers, as well as the rapidity of processing the livers. This alternative procedure was developed as several problems were encountered with the earlier procedures described by Buchanan and co-workers. (1) Their preparation of the acetone powder requires use of 19 L of acetone at −20 °C for 4.5 kg of chicken. The problems with the ventilation of these acetone vapors in a cold room were insurmountable. This step was therefore eliminated. (2) In our hands, FGAM synthetase activity either did not bind to the DE-52 column or, when it did bind, was eluted as multiple peaks. In fact, French et al. (1963) studying the *Salmonella typhimurium* FGAM synthetase reported similar problems with multiple peaks of activity. Therefore, a DEAE-Sephadex A-50 replaced the DE-52 anion-exchange columns. The method we now report is rapid and has been quite reproducible. Furthermore, the last hydroxylapatite column can be replaced by either a sizing column such as Sephacryl G-200 or a cation-exchange column such as CM-Sephadex C-50 to produce protein of specific activity ranging from 0.10 to 0.20 μmol min^{-1} mg^{-1} (Figure 1, lanes 1–3).

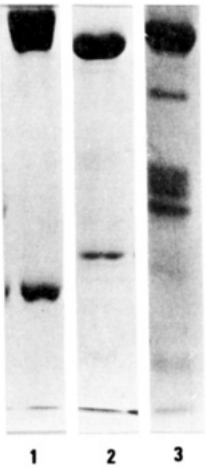


FIGURE 1: A 7.5% SDS gel of FGAM synthetase isolated as described under Materials and Methods: (lane 1) 60 μg of protein from the hydroxylapatite column; (lane 2) 35 μg of protein from the Sephacryl S-200 column; (lane 3) 52 μg of protein from the CM-Sephadex 50 column. These correspond to the three isolation procedures shown in Table I.

Stability of FGAM Synthetase. This enzyme is reasonably selective about its environment. Mizobuchi and Buchanan in 1968 reported that the enzyme in Tris buffer at pH 7.2 lost 40% of its activity in 100 min at 0 °C; we have made similar observations with HEPES buffer (pH 7.2). The higher the pH, the more rapid the loss in activity. The enzyme is therefore routinely stored in 20 mM phosphate (pH 6.5). Furthermore, as reported by Ohnoki et al. (1977), 1 mM glutamine and 10% glycerol also greatly stabilize FGAM synthetase activity. The enzyme stored under these conditions retains full activity after 2 months at −30 °C and is routinely stored in small aliquots due to loss of activity during freezing and thawing.

Synthesis and NMR Characterization of FGAR. Several chemical syntheses of FGAR have been previously reported (Carrington, 1965; Chu & Henderson, 1970). We have used a modification of the procedure of Chu and Henderson to prepare FGAR and a variety of FGAR analogues. This procedure (Scheme I) produces a 1:1 mixture of α- and β-anomers. Furthermore, the assignment by Chu and Henderson of the downfield doublet at 5.70 ppm to the β-anomeric proton of FGAR and the upfield doublet at 5.40 ppm to the α-

[3] Buchanan's units have been defined as "that amount which causes a change of 0.1 of an absorbance unit at 500 nm per 20 min incubation in the AIR synthetase assay." One such unit is equivalent to 0.00029 standard unit. Therefore, pure protein with specific activity of 380 units is equivalent to 0.12 μmol/min.

Table II: Anomeric Chemical Shifts of FGAR[a]

compd	H-1′ (ppm)	$J_{1'-2'}$ (Hz)	formyl (ppm)
FGAR[b]	5.08, 5.37	5.3, 4.6	7.89
FGAR[c]	5.09	5.3	7.85
FGAR[d]	5.39	4.5	7.86

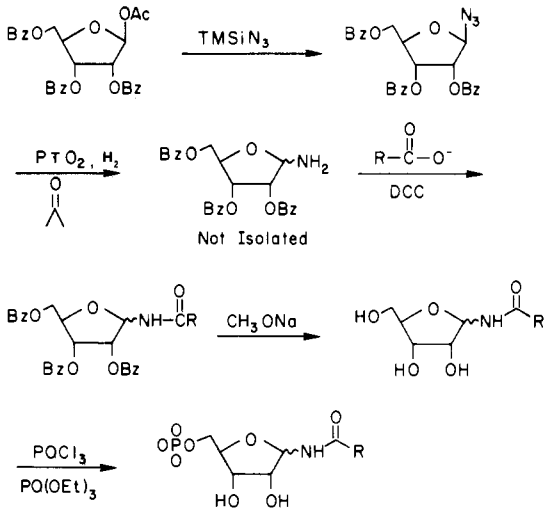
[a] Chemical shifts are referenced to HOD at 4.40 ppm. [b] FGAR prepared by the synthetic method described under Materials and Methods. [c] FGAR prepared by the biosynthetic method described under Materials and Methods. This material is enzymatically active. [d] A 1:1 anomeric mixture of FGAR was incubated with FGAM synthetase. At the end of the reaction, FGAM was separated from remaining FGAR, which was not a substrate for the synthase.

anomeric proton of FGAR is incorrect (Table II). We have assigned the upfield anomeric proton to β-FGAR on the basis of its identity with the β-FGAR we have prepared biosynthetically by modifications of the procedure of Lukens and Flaks (1963). Furthermore, incubation of αβ-FGAR with FGAM synthetase results in recovery of 0.5 equiv of FGAM and 0.5 equiv of unreacted α-FGAR (downfield anomeric proton) (Table II).

Preparation of FGAR Analogues. Early studies by Mizobuchi et al. (1968) reported that FGAR, ATP, and FGAM synthetase formed a complex, isolable by Sephadex G-25 chromatography, with a half-life of 62 min at 0 °C. We hoped to take advantage of this unique complex formation by making an FGAR affinity column that in the presence of ATP would allow rapid isolation of the protein. Furthermore, Li and Buchanan (1971) reported that *N*-(bromoacetyl)glycinamide was a potent inactivator of FGAM synthetase. Therefore, to investigate the nature of the FGAR binding site, to design a potential affinity ligand, and to prepare active site directed affinity labels, we undertook the synthesis of a variety of FGAR analogues (Table III). The general synthetic approach is analogous to that of Chu and Henderson and is indicated in Scheme I.

Interestingly, the synthetic procedure outlined in Scheme I produces an αβ (1:1) anomeric mixture of nucleosides. These are easily separable by low-pressure silica gel chromatography at the benzoylated sugar stage. Removal of the blocking groups allows isolation of pure α- and β-nucleoside. A wide variety of phosphorylating agents have been investigated for conversion of the nucleoside to nucleotide. The only method that worked successfully, however, was the one described by Chu and Henderson, a modification of the procedure of Yoshikawa et al. (1967), using a 10-fold excess of $POCl_3$. Under these conditions the anomerically pure nucleosides are converted to a 1:1 anomeric mixture of nucleotides.

Scheme I



The proton NMR data of these analogues are summarized in Table III. The downfield anomeric proton of all the FGAR analogues possesses a smaller 1′–2′ coupling constant and has been assigned to the α-anomeric configuration. This assignment is based on the comparison of the anomeric region of β-GAR and β-FGAR prepared biosynthetically with a 1:1 mixture of αβ-GAR and αβ-FGAR prepared synthetically. In both cases, the downfield, biochemically inactive, α-anomer possesses a smaller 1′–2′ coupling constant than that observed for the biochemically active upfield β-anomer.

The ^{13}C NMR data of these analogues are summarized in Table IV. The assignment of ^{13}C resonances is based on the established order of C-1′, C-4′, C-3′, C-2′, and C-5′ in the direction of increasing field with the assignments of Chettur and Benkovic (1977) for αβ-GAR (IV) and uridine 5′-monophosphate as reference material (Dorman & Roberts, 1970). Interestingly, in the case of the α isomer the normal C-1′ and C-4′ relationship is reversed with C-4′ being located downfield from the C-1′. Assignments of C-4′ are easily made due to coupling with the phosphate moiety.

Compounds I–IX were incubated with FGAM synthetase and assayed with either the [γ-^{32}P]ATP and [^{32}P]P_i determination or the glutamate dehydrogenase and 3-acetylpyridine adenine dinucleotide reduction assay described under Materials and Methods. The results of the [^{32}P]P_i assay are summarized in Table V. Three of the compounds (R = CH_2OH, CH_3,

Table III: Anomeric Chemical Shifts of FGAR Analogues[a]

compd	R	anomer	H-1′ (ppm)	$J_{1'-2'}$ (Hz)	methyl (ppm)	other (ppm)
I	CH_3	β	5.12	5.2	1.82	
		α	5.38	4.8	1.89	
II	CH_2OH	β	5.08	5.0		
		α	5.32	4.6		
III	CH_2Cl	β	5.01	4.8		
		α	5.28	4.4		
IV	$CH_2NH_3^+$	β	5.09	5.3		
		α	5.38	4.4		
V	CH_2NHCHO	β	5.08	5.3		
		α	5.37	4.6		7.89
VI	$CH_2NHCOCH_3$	β	5.05	5.3	1.62	
		α	5.32	4.5	1.65	
VII	$CH_2NHCOCH_2Cl$	β	5.08	5.2		
		α	5.37	4.4		
VIII	$CH_2NHCOOCH_2Ph$	β	5.15	4.4		
		α	5.40	4.0		
IX	L-$CHCH_3NHCHO$	β	5.08	5.0	1.00	7.68
		α	5.32	4.5		

[a] Chemical shifts are referenced to HOD at 4.40 ppm.

Table IV: ^{13}C Chemical Shifts of FGAR Analogues

compd[a]	R	C-1′ (ppm)	C-2′ (ppm)	C-3′ (ppm)	C-4′ (ppm)	$J_{C\text{-}4'\text{-}P}$ (Hz)	C-5′ (ppm)	$J_{C\text{-}5'\text{-}P}$ (Hz)	amide (ppm)	methylene (ppm)	other (ppm)
I	CH_3	82.55	70.20[c]	72.97	82.39	8.3	63.00[d]	3.85	174.57		21.43
		79.36	69.28	70.25[c]	80.44	8.3			174.13		21.39
II	CH_2OH	82.53	70.20[c]	72.96	82.34	9.6	63.09	3.0	175.36	60.28	
		78.72	69.32	69.98[c]	81.46	8.2			174.86		
III	CH_2Cl	83.28	69.64[c]	69.98	81.46	8.4	64.45	4.6	169.75	41.69	
		79.66	69.24	72.88	80.27	8.2			169.33	41.53	
IV[b]	CH_2NH_3	83.30	70.75[c]	73.64	83.15	8.6	63.53	3.3	173.47	42.62	
		79.76	69.85	70.75[c]	81.36	8.6			174.73	42.42	
V	CH_2NHCHO	83.54	70.73[c]	73.66	83.15	8.3	63.62	2.9	171.66	41.10	
		79.92	69.86	70.81[c]	81.53	8.2			171.34	40.96	164.71
VI	$CH_2NHCOCH_3$	82.80	69.99[c]	70.09	82.07	7.9	63.41	3.7	171.26	41.91	20.96
		79.31	69.21	72.91[c]	80.71	7.9			171.65	41.72	174.09
VII	$CH_2NHCOCH_2Cl$	83.39	73.53[c]	70.56	82.74	9.0	64.02			41.67	20.91
		79.89	70.33	69.93[c]	81.31	6.0				42.53	
VIII	$CH_2NHCO_2CH_2Ph$	83.12	70.32[c]	70.49	82.62	8.7	63.01	5.1	173.21	43.32	128.88
		79.40	69.45	73.28[c]	81.33	8.3			173.63	43.05	156.71
										66.72	

[a] ^{13}C NMR spectra were taken at 50 MHz with broad-band proton decoupling. Chemical shifts are referenced to an external standard dioxane at 66.50 ppm. All samples were run in D_2O at pD 6.0. For each compound, the top row represents the chemical shifts of the β compound while the second row represents the chemical shifts of the α compound. [b] GAR has previously been prepared by Chettur and Benkovic (1977). [c] The chemical shift of the C-2′ of the β-isomer is not distinguishable from the chemical shift of the C-3′ of the α-isomer. In some cases an anomeric distribution of α:β that varied significantly from 1:1 allowed the assignment to be made. [d] Only one peak was observed for the C-5′ carbon of both α- and β-anomers.

Table V: ATPase Activity of FGAR Analogues with FGAM Synthetase

compd	R	K_m (mM)	V/K (rel)	V_{max} (rel)
β-V	CH_2NHCHO[a]	0.23	100	100
α/β-V	CH_2NHCHO[b]	0.18	124	99
II	CH_2OH[c]	2.4	4.6	48
VI	$CH_2NHCOCH_3$[c]	6.6	0.65	20
I	CH_3[c]	2.3	0.44	4.5
III	CH_2Cl			
IV	CH_2NH_3			
VII	$CH_2NHCOCH_2Cl$			
VIII	$CH_2NHCO_2CH_2Ph$			
IX	L-$CHCH_3NHCHO$			

[a] Prepared biosynthetically. [b] Prepared by the chemical procedure as described under Materials and Methods. [c] This table indicates that neither the K_m nor V_{max} value of a 1:1 mixture of α/β-FGAR differs significantly from the value obtained with β-FGAR. For the analogues in Table V, the R concentrations represent β-anomers only and have been determined as described under Materials and Methods. The assumption has been made that the α-anomer does not bind.

and $CH_2NHCOCH_3$) exhibited ATPase activity that varied from 5 to 50% the rate of P_i production observed with FGAR. All of these compounds had K_m values substantially larger than that of FGAR itself. Compounds I, II, and VI were therefore further investigated to determine if they also caused glutaminase activity. Only compound VI caused glutaminase activity, and the rate of glutamate production catalyzed by FGAM synthetase was in fact equivalent to the rate of P_i production under identical assay conditions.

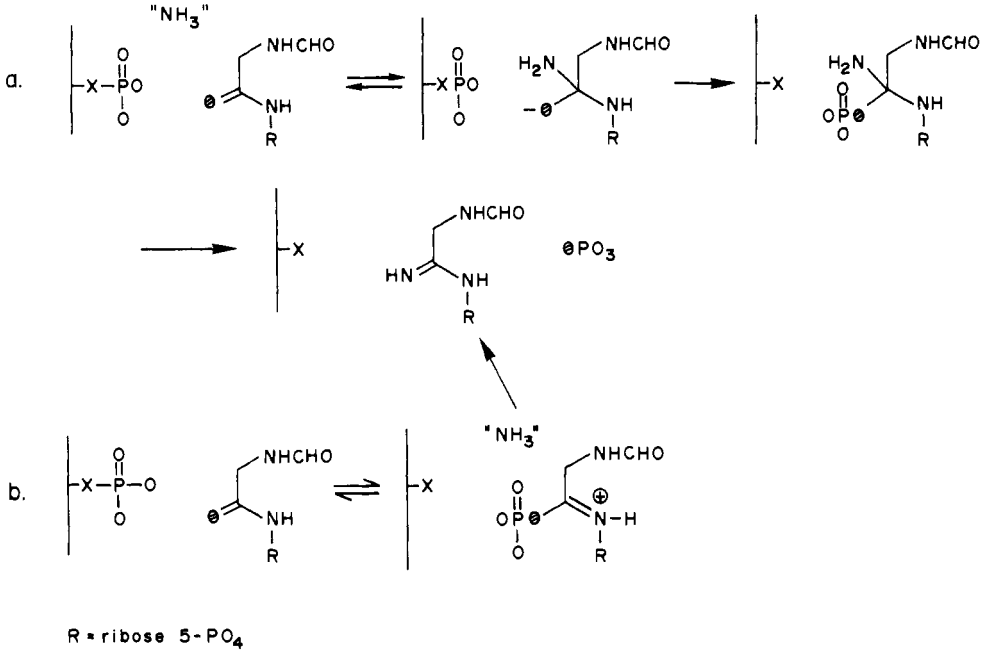
These results strongly suggested that FGAM synthetase was also catalyzing the conversion of the amide of VI to the amidine of VI. This possibility was investigated by incubating [γ-^{32}P]ATP, VI, and FGAM synthetase at 37 °C until 200 nmol of [^{32}P]P_i was produced. The pH of the solution was then adjusted to 5, and this was applied to a DEAE-Sephadex A-25 column equilibrated in 10 mM ammonium acetate (pH 5.0). Under these conditions, the amidine of VI should be uncharged and pass directly through the column, while the amide of VI should possess one negative charge and be retained by the column. The material that passed directly through the column which contained equal amounts of ribose, based on the orcinol test, and phosphate was then adjusted to pH 8 and applied to a second DEAE anion-exchange column (previously equilibrated at pH 8). The material was retained on the column and eluted with 200 mM ammonium acetate (pH 8.0) to give 65% (130 nmol) of material that retained a 1:1 ratio of phosphate and ribose. Identical results were obtained by using FGAR in place of VI with a similar 65% recovery of FGAM. The FGAM was unequivocally identified by quantitative conversion to AIR by incubation with ATP and AIR synthetase from *E. coli*. Unfortunately, the amidine of VI does not appear to be a substrate for AIR synthetase from either *E. coli* or chicken liver.

Furthermore, it is interesting to note that incubation of VI, ATP, and FGAM synthetase in the absence of glutamine allows isolation through Sephadex G-50 chromatography of a complex with 0.3 mol of ^{32}P/mol of protein. An analogous experiment with FGAR replacing VI produced 0.5 mol of ^{32}P/mol of protein. Unfortunately, due to the high K_m value of VI, it is not possible to saturate protein in VI, most reasonably accounting for low stoichiometry. Similar experiments with compounds I and II indicated no complex formation. Hence, complex formation appears to be an indication of the ability of the analogue to be a substrate for FGAM synthetase.

Compounds III, IV, VII, VIII, and IX (Table V) upon incubation with [γ-^{32}P]ATP and FGAM synthetase produced no [^{32}P]P_i even when concentrations of enzyme 100 times that normally employed were used. The concentrations of these analogues required to measure K_i values would have been in the high millimolar range, and therefore, these compounds were not investigated further. Li and Buchanan in 1971 reported that *N*-(bromoacetyl)glycinamide ($NH_2COCH_2NHCOCH_2Br$) was an irreversible inhibitor of FGAM synthetase. Therefore, both compounds III (R = CH_2Cl) and VIII (R = $CH_2NHCOCH_2Cl$) were examined as potential time-dependent inactivators or affinity labels for FGAM synthetase. Unfortunately, no time-dependent inactivation was observed in the presence or absence of glutamine. This suggests that the glycine derivative investigated by Li and Buchanan might be alkylating the cysteine moiety in the glutamine binding site by a similar mechanism to that evoked for iodoacetate (Ohnoki et al., 1977).

A number of conclusions may be drawn from these investigations. (1) On the basis of the high specificity of the enzyme for the formylglycine moiety, the use of an FGAR analogue

Scheme II



affinity column linked through its glycine side chain in the presence of ATP to isolate FGAM synthetase is not viable. (2) FGAM synthetase is very specific for the side-chain formylglycine moiety with only the *N*-acetylglycine derivative being converted to an amidine. (3) An uncoupling of ATPase and glutaminase activity is observed with R = CH_2OH and CH_3. This phenomenon has precedence based on the work of Griffith and Meister (1981) on 5-oxo-L-prolinase. In this case, the coupling between the cleavage of the amide bond of 5-oxoproline and that of ATP hydrolysis may be partially or completely lost if either substrate is modified structurally. The mechanism of ATPase catalyzed by FGAM synthetase with the majority of FGAR analogues remains to be established.

Several mechanisms for the hydrolysis of ATP in the presence of I (FGAR analogue, R = CH_3) can be envisioned. The ATP or phosphorylated enzyme intermediate can be attacked by H_2O directly to produce P_i. Alternatively, the ATP can phosphorylate the FGAR analogue I, and subsequent attack by H_2O in place of NH_3 (Scheme IIb) would transfer the amide oxygen of I to the P_i. In an attempt to distinguish between these mechanisms, we incubated I, FGAM synthetase, and ATP in a 1:1 mixture of $H_2{}^{16}O/H_2{}^{18}O$. After substantial turnover, the P_i was isolated and the ^{31}P NMR spectrum recorded. The spectrum indicated enzymatic production of a 1:1 mixture of $[^{16}O]P_i$ to $[^{18}O]P_i$. This result strongly suggests that a phosphorylated intermediate of I does not account for the ATPase activity.[4] More importantly, this result demonstrates that the mechanism of ATP hydrolysis catalyzed by FGAM synthetase and induced by I differs from that with the normal substrate FGAR.

Conversion of $[^{18}O]$*FGAR to* $[^{18}O]P_i$. Several mechanisms may be proposed for the ATP- and glutamine-dependent conversion of FGAR to FGAM (Scheme II).[5] Scheme IIa is a modification of a mechanism proposed by Mizobuchi et al. (1968) involving attack of NH_3 of FGAR to form the carbinolamine, which is then phosphorylated by ATP with subsequent release of phosphate. Alternatively, Scheme IIb postulates a mechanism that involves a phosphorylated FGAR intermediate which might be formed in the presence or absence of ammonia. This type of mechanism has been previously proposed by Westheimer and his co-workers on the basis of elegant model studies (Westheimer, 1981; Satterthwait & Westheimer, 1980). Moreover, recent investigations by von der Saal et al. (1985) using positional isotope exchange methodology support this type of mechanism for the enzyme cytidine-5′-triphosphate synthetase. In addition to the phosphorylated FGAR intermediate, a phosphorylated enzyme intermediate is also indicated in Scheme II. This is based on a report by Mizobuchi et al. (1968) of ATP/ADP exchange, a finding we have recently substantiated and are investigating in more detail. Unfortunately, ATPγS, while a linear competitive inhibitor of ATP (K_{is} = 0.69 mM), is not a substrate for FGAM synthetase, and therefore, the phosphorus stereochemistry methodology would not be of use to establish the existence of a phosphorylated enzyme intermediate (Gerlt et al., 1983).

Both mechanisms of Scheme II indicate that the ^{18}O from $[^{18}O]$FGAR should be transferred to inorganic phosphate. To test this prediction, $[^{18}O]$glycine (>95% labeled) prepared by the procedure of Mears and Sobotka (1939) was diluted with 1 equiv of glycine and incorporated into FGAR by the biosynthetic procedure described under Materials and Methods. The 1:1 $[^{18}O/^{16}O]$FGAR was incubated with FGAM synthetase and the phosphate isolated by anion-exchange chromatography. The ^{31}P NMR spectrum of the isolated P_i is shown in Figure 2 and indicates the perturbation of ^{18}O on the chemical shift of the ^{31}P nucleus (Cohn & Hu, 1979). ^{18}O is transferred quantitatively from the amide oxygen of FGAR to inorganic phosphate as predicted by both mechanisms.

Summary. A new rapid isolation of the protein FGAM synthetase is reported that avoids the problems of preparation of acetone powder and the inconsistencies of the DE-52 anion-exchange column. This preparation has allowed us to establish that the *N*-acetylglycine derivative of FGAR is an alternate substrate for this protein but that the enzyme is highly specific for its formylglycine side-chain moiety. Several α-halo amide FGAR analogues designed as potential affinity

[4] A mechanism in which H_2O attacks the phosphate moiety of phosphorylated I cannot be ruled out; however, experiments with $[^{18}O]$FGAR rule out this possibility during the normal reaction.

[5] A concerted mechanism cannot be ruled out as proposed by a reviewer.

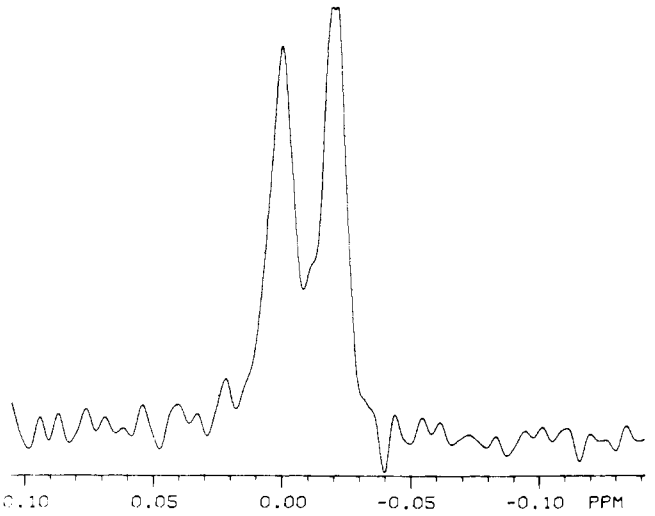


FIGURE 2: An 80-MHz ^{31}P NMR spectrum of the P_i isolated from the reaction of FGAM synthetase, ATP, glutamine, and 1:1 [^{16}O/^{18}O]FGAR.

labels were ineffective. [^{18}O]FGAR in the presence of FGAM synthetase transferred its amide oxygen to P_i, consistent with proposed mechanisms for the role of ATP in activating the leaving group.

Registry No. 1, 7408-41-5; I, 100838-27-5; β-I, 100763-83-5; α-I, 100763-84-6; II, 100763-94-8; β-II, 100992-00-5; α-II, 100763-85-7; III, 100895-66-7; β-III, 100763-86-8; α-III, 100763-87-9; IV, 92379-77-6; β-IV, 10074-18-7; α-IV, 73650-37-0; V, 6159-80-4; β-V, 349-34-8; VI, 100838-28-6; β-VI, 100789-91-1; α-VI, 100763-88-0; VII, 100838-29-7; β-VII, 100763-89-1; α-VII, 100763-90-4; VIII, 100838-30-0; β-VIII, 100763-91-5; α-VIII, 100763-92-6; IX, 100838-31-1; β-IX, 100789-92-2; α-IX, 100763-93-7; FGAM, 6157-85-3; FGAR, 349-34-8; ATPase, 9000-83-3; formylglycinamidine synthetase, 9032-84-2; glutaminase, 9001-47-2; *N*-(hydroxyacetyl)-2,3,5-tri-*O*-benzoyl-D-ribofuranosylamine, 100763-95-9; imidazole, 288-32-4; *tert*-butyldimethylsilyl chloride, 18162-48-6; [^{13}C,^{18}O]formylglycinamide ribonucleotide, 100763-96-0.

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