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Formylglycinamide Ribonucleotide Synthetase from *Escherichia coli*: Cloning, Sequencing, Overproduction, Isolation, and Characterization[†,‡]

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ABSTRACT: The *pur*L gene of *Escherichia coli* encoding the enzyme formylglycinamidine ribonucleotide (FGAM) synthetase which catalyzes the conversion of formylglycinamide ribonucleotide (FGAR), glutamine, and MgATP to FGAM, glutamate, ADP, and P_i has been cloned and sequenced. The mature protein, as deduced by the structural gene sequence, contains 1628 amino acids and has a calculated M_r of 141 418. Comparison of the *pur*L control region to other pur loci control regions reveals a common region of dyad symmetry which may be the binding site for the "putative" repressor protein. Construction of an overproducing strain permitted purification of the protein to homogeneity. N-Terminal sequence analysis and comparison of glutamine binding domain sequences (Ebbole & Zalkin, 1987) confirm the amino acid sequence deduced from the gene sequence. The purified protein exhibits glutaminase activity of 0.02% the normal turnover, and NH_3 can replace glutamine as a nitrogen donor with a K_m = 1 M and a turnover of 3 min^{-1} (2% glutamine turnover). The enzyme forms an isolable (1:1) complex with glutamine: $t_{1/2}$ is 22 min at 4 °C. This isolated complex is not chemically competent to complete turnover when FGAR and ATP are added, demonstrating that ammonia and glutamine are not covalently bound as a thiohemiaminal available to complete the chemical conversion to FGAM. Hydroxylamine trapping experiments indicate that glutamine is bound covalently to the enzyme as a thiol ester. Initial velocity and dead-end inhibition kinetic studies on FGAM synthetase are most consistent with a sequential mechanism in which glutamine binds followed by rapid equilibrium binding of MgATP and then FGAR. Incubation of [^{18}O]FGAR with enzyme, ATP, and glutamine results in quantitative transfer of the ^{18}O to P_i.

Formylglycinamide ribonucleotide (FGAM)[1] synthetase is the fourth step in the purine biosynthetic pathway and catalyzes the irreversible conversion of formylglycinamide ribonucleotide (FGAR), glutamine, and ATP to FGAM, P_i, ADP, and glutamate (eq 1). This protein was first purified to homogeneity from *Salmonella typhimurium* by French et al.

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[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02848.

[1] Abbreviations: FGAM, formylglycinamidine ribonucleotide; FGAR, formylglycinamide ribonucleotide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; AIR, aminoimidazole ribonucleotide.

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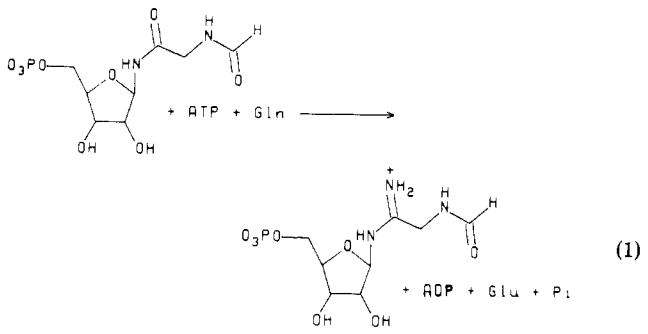

(1)

in 1963 and then from chicken liver by Mizobuchi and Buchanan in 1968. Recently, Ebbole and Zalkin (1987) reported the cloning and characterization of a 12-gene cluster in *Bacillus subtilis* encoding nine enzymes for the purine biosynthetic pathway including the gene, *purL*, for FGAM synthetase. The proteins from *S. typhimurium* and chicken liver are monomers of M_r = 135K and 133K, respectively. This contrasts with results from the gene sequence of *B. subtilis* (identified by sequence homology with the *E. coli* protein reported in this paper) which indicate that FGAM synthetase is composed of two polypeptides: M_r = 24 755 (*pur*Q gene) and 80 300 (*pur*L gene).

Extensive studies from Buchanan's laboratory on the chicken liver protein have provided much insight about the nature of the glutamine binding domain (Buchanan, 1973) and have provided some thought-provoking information about the properties of the ATP and FGAR binding site(s). Specifically, Mizobuchi et al. (1968) made the unusual observation that chicken liver FGAM synthetase forms a stable isolable complex with glutamine, $t_{1/2}$ = 125 min at 4 °C, and a stable isolable complex with FGAR and ATP, $t_{1/2}$ = 62 min at 4 °C.

Recent efforts have focused on unraveling a generalized mechanism by which enzymes in the purine [aminoimidazole ribonucleotide (AIR) synthetase] and pyrimidine (CTP synthetase) pathways convert amides to amidines using ATP as a dehydrating agent (Schendel & Stubbe, 1986; Schrimsher et al., 1986; von der Saal et al., 1985; Westheimer, 1981) and by which enzymes from these pathways convert glutamine to a "putative NH_3 equivalent" (Zalkin, 1985).

The unusual stability of the FGAM synthetase–substrate complexes reported for the chicken liver enzyme might provide a opportunity to examine the detailed mechanism if these complexes are chemically and kinetically competent in the presence of the additional required substrate(s).

Initially we focused our efforts on obtaining large amounts of the chicken liver protein for use in detailed mechanistic studies. Unfortunately, instability of the protein as well as lack of reproducibility of isolation of the protein, in analogy with problems reported by Mizobuchi and Buchanan (1968) and French et al. (1963), made an alternative source of the protein desirable.

The availability of *Escherichia coli pur*L mutants has provided us with this alternative source. The present paper reports the successful cloning behind the λpL promoter and sequencing of the gene for FGAM synthetase and overexpression of the protein. FGAM synthetase subsequent to heat induction is 50% of the total protein (approximately 150 times that found in wild-type *E. coli*), which has greatly facilitated rapid isolation or large amounts of homogeneous protein. The kinetic mechanism of this protein has been elucidated by initial velocity studies in conjunction with product and dead-end inhibition studies. A detailed study of the properties of the FGAM synthetase–glutamine complex is also reported. These studies provide the foundation for future detailed investigations on the regulation of gene expression and on the mechanism of conversion of FGAR to FGAM.

Materials and Methods

Sephadex G-25, Sephadex G-50, Sephadex G-150, Sephacryl S-200, DEAE-Sephadex A-25 and A-50, phosphoenolpyruvic acid, ATP, ampillicin, L-glutamine, rabbit muscle pyruvate kinase (355 units mg^{-1}), rabbit muscle L-lactate dehydrogenase (920 units mg^{-1}), *E. coli* glutaminase (8.4 units mg^{-1}), bovine serum albumin, and molecular weight standards for gel filtration and gel electrophoresis were obtained from Sigma Chemical Co. Nitrocellulose, DEAE-agarose Bio-Gel A, hydroxylapatite HPT, goat anti-rabbit IgG–horseradish peroxidase conjugate, Tween-20, and horseradish peroxidase color reagent were purchased from Bio-Rad. Cellulose F thin-layer chromatography plates were obtained from J. T. Baker Chemical Co. [1-^{13}C]Glycine (90.0 atom % ^{13}C) was obtained from MSD Isotopes. $H_2{}^{18}O$ (95.1 atom % ^{18}O) was purchased from Monsanto Research Corp. [U-^{14}C]-L-Glutamine (283 mCi/mmol) was purchased from New England Nuclear. [1-^{13}C,$^{18}O_2$]Glycine was prepared by the procedure of Mears and Sobotka (1939). FGAR was synthesized by the procedure of Schendel and Stubbe (1986). Concentrations of protein solutions were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. L-Glutamine concentrations were determined by measuring the ammonia released or the glutamate formed by the action of glutaminase (Williamson & Corkey, 1979). AIR synthetase (3 units mg^{-1}) was isolated from *E. coli* TX393/pJS24 by the procedure of Schrimsher et al. (1986a). ^{13}C NMR spectra were obtained at 50.3 MHz, and ^{31}P NMR spectra were obtained at 80.9 MHz on a Nicolet 200-MHz broad-band spectrometer. All solutions were analyzed for radioactivity in Packard Scint-A scintillation fluid with a Packard 310 liquid scintillation counter. Protein sequencing was done according to the automated Edman degradation procedure by the University of Wisconsin Biotechnology Center.

Strains and Media. Strain TX160 [(Δ*lac*) *pur*L213::*lac*ZY::λpl(209)] was created from strain TX40 (Smith & Gots, 1980) by the procedure of Casadaban (Groisman et al., 1984). Strain TX540 [Δ(*lac*) *pur*L213::*lac*ZY::λpl(209)*srl*C300::Tn10 *rec*A56] was constructed and employed for complementation analysis. Strain JM83 (Messing, 1979) was used as a recipient to identify subclones containing restriction fragments from the *pur*L region while strain JM101 (Messing, 1979) was employed for the propagation of M13 bacteriophages. Strain TX635 (F′ *lac*Z$^+$ cI857; Mieschendahl & Müller-Hill, 1985) contains an episome-borne temperature-sensitive λ repressor and was used as a host for the λpL plasmids. Strains were made competent and transformed by the procedure of Dagert and Ehrlich (1979). The minimal medium of Neidhart et al. (1974) and the rich media described by Miller (1972) were used for the growth of the *E. coli* K12 strains. The recombinant DNA techniques employed have been described (Tiedeman et al., 1985).

Purification of E. coli FGAM Synthetase. E. coli strain TX635/pJS113 was grown at 30 °C in LB media (Mantiatis, 1982) in house distilled water supplemented with 1 mL/L Vogel-Bonner (1956) trace minerals. Bacteria were grown to an OD_{600} = 0.95 (doubling time, 1 h), heat induced by an equal volume of LB media at 56 °C, and grown at 42 °C for an additional 3 h. The bacteria were harvested, and the cells were frozen with liquid nitrogen and stored at −80 °C. The purification of FGAM synthetase was carried out at 4 °C, and all buffers used contained 1 mM glutamine and 5 mM β-mercaptoethanol.

The cells (1 g) were resuspended in 5 volumes (5 mL) of 50 mM potassium phosphate, pH 6.8, and broken in a French press pressure cell at 12 000 psi, and the extract was centrifuged at 17000*g* for 20 min. The supernatant was adjusted to 40% saturation in ammonium sulfate by the slow addition of solid ammonium sulfate (0.243 g/mL). After the ammonium sulfate had dissolved, the solution was stirred for 20 min and then centrifuged at 17000*g* for 10 min. The supernatant was then brought to 55% saturation of ammonium sulfate by the addition of solid ammonium sulfate (0.097 g/mL). The solution was again stirred for 20 min and then centrifuged at 17000*g* for 10 min. The pellet was redissolved in a minimal volume (0.8 mL) of 50 mM potassium phosphate, pH 6.8, and then desalted on a Sephadex G-25 column (2 × 15 cm) equilibrated in the same buffer. The fractions containing protein were collected and applied to a column of DEAE-agarose (1.5 × 8.5 cm) equilibrated in 50 mM potassium phosphate, pH 6.8. The column was washed until the absorbance at 280 nm was less than 0.01. The FGAM synthetase was then eluted with 50 mM potassium phosphate (pH 6.8), 75 × 75 mL linear gradient from 0 to 150 mM KCl. The fractions containing activity were pooled and concentrated to less than 5 mL in an Amicon ultrafiltration apparatus using a PM-30 membrane. The enzyme, quick frozen in 50 mM potassium phosphate, pH 6.8, containing 25% (v/v) glycerol, was stored at −80 °C and was stable for at least 2 weeks.

Native and Subunit Molecular Weight of FGAM Synthetase. A column of Sephadex G-150 was equilibrated with 50 mM potassium phosphate, pH 6.8, and calibrated with blue dextran (average M_r of 2×10^6) and proteins of known molecular weight. The proteins used were yeast alcohol dehydrogenase (M_r = 150 000), conalbumin (M_r = 78 000), ovalbumin (M_r = 45 000), bovine erythrocyte carbonic anhydrase (M_r = 29 000), and horse heart cytochrome *c* (M_r = 12 400). The native M_r of FGAM synthetase was obtained from a plot of M_r vs K_{ave}. Subunit molecular weight was determined from SDS–polyacrylamide gel electrophoresis (7.5% acrylamide) performed according to the procedure Laemmli (1970). The molecular weight standards used included *E. coli* β-galactosidase (M_r = 116 000), rabbit muscle phosphorylase B (M_r = 91 100), bovine serum albumin (M_r = 60 000), egg albumin (M_r = 44 000), and bovine erythrocyte carbonic anhydrase (M_r = 29 000).

Enzyme Assays. FGAM synthetase was assayed by one of three methods. (1) The amount of FGAM produced was determined according to a coupled assay procedure with AIR synthetase (Schendel & Stubbe, 1986) in which the buffer was 50 mM HEPES (pH 7.25). (2) The formation of ADP was quantitated spectrophotometrically by a coupled assay procedure with pyruvate kinase and lactate dehydrogenase. A typical reaction mixture contained in a final volume of 1 mL 40 mM HEPES (pH 7.25), 20 mM $MgCl_2$, 10 mM KCl, 1 mM L-glutamine, 2 mM ATP, 0.2 mM α/β-FGAR, 0.2 mM NADH, 1.0 mM phosphoenolpyruvate, 3.5 units of pyruvate kinase, and 4.7 units of lactate dehydrogenase, preincubated at 37 °C. The reaction was started by the addition of FGAM synthetase (0.02 unit). The initial velocities were calculated from the decrease in absorbance at 340 nm with $\epsilon = 6200\ M^{-1}\ cm^{-1}$. (3) The formation of glutamate was determined according to a coupled assay procedure with glutamate dehydrogenase in a stepwise manner (Schendel & Stubbe, 1986). One unit of enzymatic activity is defined as the amount of enzyme required to produce 1 μmol of product min^{-1} at 37 °C.

Kinetic Analysis. All kinetic studies were conducted in the presence of 40 mM HEPES (pH 7.25), 20 mM $MgCl_2$, and 10 mM KCl. The Michaelis constants for FGAR, MgATP, and glutamine were determined by varying the concentration of one substrate at saturated levels of the other two and fitting the data with nonlinear least-squares regression analysis to eq 2. Initial velocity data were obtained by varying the concentration of two substrates from $0.2K_m$ to $5K_m$ while holding the third substrate concentration constant ($1.5K_m$) or by varying the concentration of one substrate at a fixed ratio of the other two (Fromm, 1967; Chu & Henderson, 1972). The intial velocity data were fit to eq 3 or the corresponding equation with the appropriate terms missing, to determine the best steady-state kinetic model (Viola & Cleland, 1982). Inhibition studies were analyzed with the computer programs of Cleland (1979). Data corresponding to competitive, noncompetitive, or uncompetitive inhibition were fit to eq 4–6.

$$v = VA/[K_a + A] \quad (2)$$

$$v = VABC/[\text{const} + (\text{coeff A})A + (\text{coeff B})B + (\text{coeff C})C + K_aBC + K_bAC + K_cAB + ABC] \quad (3)$$

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (4)$$

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (6)$$

Exchange Reactions. The ATP/ADP exchange reactions contained 40 mM HEPES (pH 7.2), 20 mM $MgCl_2$, 10 mM KCl, 1.4 mM MgATP, 1.2 μM [U-^{14}C]ADP (1.0×10^6 cpm/μmol), and 0.02 unit of FGAM synthetase in a final volume of 200 μL. The reaction mixture was incubated at 37 °C, and 50-μL aliquots were withdrawn at 0, 5, 10, and 20 min. These samples were added to 100 μL of 50 mM potassium phosphate, pH 4.8, containing 5 mM tetrabutylammonium bromide and immediately frozen in a dry ice–acetone bath. The samples were analyzed by reverse-phase ion-pairing HPLC on an Alltech C-18 column with a flow rate of 1 mL/min using a solvent composed of 50 mM potassium phosphate, pH 4.8, 5 mM tetrabutylammonium bromide, and 24% (v/v) methanol. The retention time for ADP was 8.5 min and for ATP was 16 min. The ATP peak was collected and concentrated to dryness in vacuo, redissolved in 1 mL of water, and analyzed for radioactivity by liquid scintillation counting.

The glutamine/glutamate exchange reactions contained 40 mM HEPES (pH 7.2), 20 mM $MgCl_2$, 10 mM KCl, 460 μM glutamine, 482 μM [U-^{14}C]glutamate (1.7×10^6 cpm/μmol), and 0.02 unit of FGAM synthetase in a final volume of 200 μL. The reactions were incubated at 37 °C, and 40-μL aliquots were withdrawn at 0, 5, 10, and 20 min. The samples were applied to a 1-mL DEAE-Sephadex A-25 column. The column was washed with 5 mL of water to elute the glutamine, and 2 mL of the eluant was analyzed for radioactivity by liquid scintillation counting.

Formation of Enzyme–Glutamine Complex. To a total volume of 300 μL containing 40 mM HEPES, pH 7.25, 10 mM KCl, 20 mM $MgCl_2$, and 3 mM [U-^{14}C]-L-glutamine (3.5×10^6 cpm/μmol) was added 1–10 nmol of FGAM synthetase, and the mixture was incubated at 37 °C for 5 min. The reaction was then applied to a Sephadex G-50 column (0.75 × 21 cm) equilibrated in 50 mM HEPES, pH 7.3. Fractions of 1 mL were collected, the absorbance at 280 nm was recorded, and 100–500 μL of each fraction was analyzed for ^{14}C by liquid scintillation counting. Protein did not cause any significant quenching during scintillation counting. The molar ratio of [U-^{14}C]glutamine to enzyme (binding ratio) was calculated with a M_r = 140K for the protein, based on the gene sequence, and 2 units mg^{-1} as the specific activity of the pure

protein. The half-life of the enzyme–glutamine complex was determined by using columns of varying lengths (0.75 × 15–45 cm) run with variable flow rates and by plotting the log of the binding ratio vs the time the complex was on the column.

Reaction of Enzyme–Glutamine Complex with Hydroxylamine. To 5.0 or 6.0 nmol of complex (isolated as discussed above) in 500 μL of 50 mM HEPES, pH 7.3, was added 500 μL of 2 M hydroxylamine, pH 7.3, or 1.0 mL of 1.5 M hydroxylamine in 6 M guanidine hydrochloride. The reactions were incubated at 25 °C for 20 min and then diluted to 20 mL and applied to DEAE-Sephadex A-25 columns (1 × 5 cm and 1 × 10 cm, respectively). The columns were washed with water, conditions to elute glutamine and γ-glutamylhydroxamate, and then with 250 mM triethylammonium bicarbonate, conditions to elute glutamate. Fractions of 1 mL were collected and analyzed for radioactivity by liquid scintillation counting. Fractions from the H_2O elution containing radioactivity were pooled, adjusted to pH 7.0, and heated to 100 °C for 7.5 min, a procedure which converts γ-glutamylhydroxamic acid to pyrrolidonecarboxylic acid (Levintow et al., 1955). To separate any resulting pyrrolidonecarboxylic acid from glutamine, the sample was diluted to 10 mL with water and applied to a DEAE-Sephadex A-25 column (1 × 5 cm). The column was washed with 10 mL of water, and eluted with 250 mM triethylammonium bicarbonate. The fractions containing radioactivity were pooled, concentrated to dryness, and utilized for further characterization.

Separation of L-*Glutamate,* L-*Glutamine, and Pyrrolidonecarboxylic Acid.* Samples isolated by the preceding protocol were spotted on cellulose F plates and developed with a solvent composed of absolute ethanol, 2-methyl-2-propanol, water, and formic acid (88%) (60:20:15:5) (Mizobuchi & Buchanan, 1968a). R_f values were as follows: L-glutamine, 0.25; L-glutamate, 0.36; pyrrolidonecarboxylic acid, 0.62. Pyrrolidonecarboxylic acid was visualized according to the starch–iodide procedure of Pan and Dutcher (1956), and amino acids were visualized with 0.3% ninhydrin in acetone. The radioactivity was quantitated by liquid scintillation counting subsequent to cutting the plate into 1-cm strips and elution of compounds with 1 mL of 200 mM triethylammonium bicarbonate.

Turnover of Enzyme–Glutamine Complex. To 6.8 nmol of complex (isolated as described above) in 600 μL of 10 mM HEPES, pH 7.3, was added 100 μL of 200 mM HEPES, pH 7.3, containing 150 mM $MgCl_2$, 75 mM KCl, 15 mM Mg-ATP, 3.5 mM FGAR, and 0.2 unit of AIR synthetase. The reaction mixture was incubated at 37 °C for 5 min, and the amount of AIR produced was determined according to a modified Bratton–Marshall assay (A_{500} nm and with ϵ = 24 600 M^{-1} cm^{-1} (Schendel & Stubbe, 1986; Schrimsher et al., 1986b). A control reaction containing 4.8 nmol of complex was run under identical conditions with those described above except 2.1 nmol of glutamine was also included. This control allows determination of the lower limit of detection of AIR by this method.

^{18}O Transfer from [^{18}O]FGAR to P_i. To a 5-mm NMR tube containing 30 mM ATP, 30 mM L-glutamine, 10 mM KCl, 50 mM $MgCl_2$, and 40 mM HEPES, pH 7.3, in a final volume of 1 mL (30% D_2O) was added 1.0 unit of FGAM synthetase. The ^{31}P NMR spectrum was taken at 80.9 MHz, and then 15 μmol of [^{18}O]FGAR was added and the reaction mixture incubated at 37 °C for 30 min. When the reaction was complete, determined by ^{31}P NMR, the reaction mixture was diluted to 3 mL and passed through an Amicon Centricon-30 ultrafiltration apparatus with a YM-30 membrane. The filtrate was collected and applied to a DEAE-Sephadex A-25 column (1.0 × 4 cm). The column was washed with 2 volumes (8 mL) of water, and the inorganic phosphate was eluted with 250 mM triethylammonium bicarbonate (pH 7.8). The fractions containing the inorganic phosphate were pooled, concentrated to dryness in vacuo, and redissolved in 0.5 mL of D_2O containing 1 mM EDTA. The sample was placed in a 5-mm NMR tube, and the ^{31}P NMR spectrum was recorded at 80.9 MHz with a sweep width of ±500 Hz, quadrature phase detection, a 90° pulse angle with a pulse delay of 2 s, and an 8K data block. To enhance resolution, exponential multiplication with a line-broading factor of 0.1 Hz was applied to the FID before Fourier transformation. The ratio of [^{16}O]/[^{18}O]P_i was determined by weighing the appropriate peaks.

Western Blot Analysis. SDS–polyacrylamide gel electrophoresis was run as described above, and the proteins were transferred to nitrocellulose electrophoretically (Burnette, 1981). Antibodies used in probing the blots were prepared in rabbits by injection of 300–500 μg of native chicken liver FGAM synthetase in Freund's complete adjuvant followed by booster injections with 300–500 μg of FGAM synthetase in Freund's incomplete adjuvant. Antibody was visualized by using a goat anti-rabbit IgG–horseradish peroxidase conjugate as described by Hawkes et al. (1982). Proteins were visualized by Coomassie blue staining for 10 s. Antibody 80 times the concentration required to detect the chicken liver enzyme indicated no cross-reactivity.

Results and Discussion

Cloning and Complementation Analysis of the purL Locus. Plasmids containing the *pur*L locus were cloned by the mini-Mu procedure of Casadaban (Groisman et al., 1984) and isolated by complementation of strain TX540. After initial characterization of several plasmids, the *pur*L locus was subcloned by partial *Sau*3A digestion into the *Bam*HI site of plasmid pUC19 (Yanisch-Perron et al., 1985). A representative subclone with an approximately 6-kb insert was designated pJS80 and used for further analysis. The limits of the *pur*L structural gene were localized by testing specific subcloned restriction fragments for complementation in strain TX540. One end of the *pur*L gene was localized between a *Mlu*I and a *Ssp*I site while the other end was localized between a *Fsp*I and a *Ssp*I site (Figure 1). This region can maximally encode a protein of approximately 150 000 daltons, sufficient to accommodate the subunit size of FGAM synthetase from *Salmonella* (French et al., 1963).

Construction of an Expression Vector for FGAM Synthetase. An expression vector for FGAM synthetase was created by reconstructing a DNA fragment spanning the *Hin*dIII site at nucleotide 1273 to the *Ssp*I site at nucleotide 5339. This fragment, lacking the *pur*L promoter, was then inserted into plasmid pJS88 (A. A. Tiedeman and J. M. Smith, unpublished results) to produce pJS113. Plasmid pJS88 is a λpL expression vector similar to the ones described by Remaut et al. (1981). Strain TX635 was used as the host, and synthesis of FGAM synthetase was induced by a temperature shift to 42 °C.

DNA Sequence Analysis. DNA sequence was determined by the dideoxy chain termination method of Sanger (Sanger et al., 1977). The 6-kb DNA fragment containing the *pur*L locus was subcloned as various separate restriction fragments into M13mp18 and overlapping deletion subclones generated by the procedure of Henikoff (1984) for DNA sequencing. The DNA sequence of the entire 6-kb DNA fragment was assembled from overlapping subclones for both strands as shown in Figure 2. The DNA sequences were compiled and

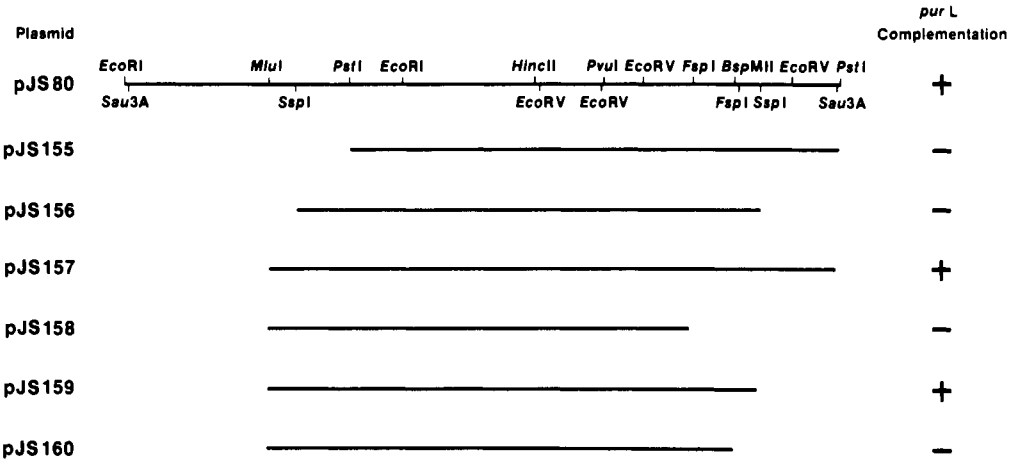

FIGURE 1: Partial restriction map and complementation analysis of the *pur*L locus. Only the major restriction sites pertinent to this study are shown. The *Sau*3A sites represent the termini of the restriction fragment while the outer *Eco*RI and *Pst*I restriction sites show the orientation within the cloning region of pUC19. Complementation studies were carried out in strain TX540.

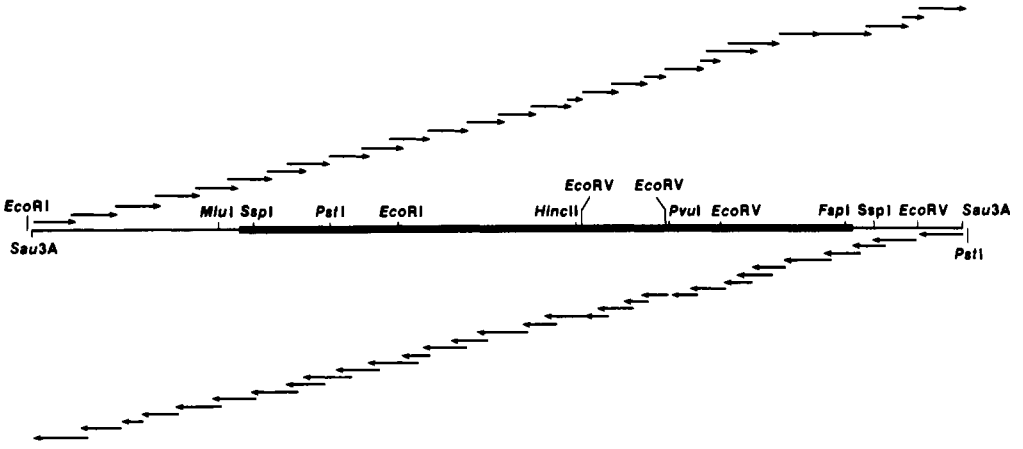

FIGURE 2: Sequencing strategy for *pur*L. The location of the 6-bp restriction enzyme sites used in subcloning and sequencing studies are indicated. The arrows denote sequencing direction, and the length of the arrow is proportional to the number of nucleotides determined. The *pur*L coding region extends from nucleotide 1294 to nucleotide 5178 and is indicated by the thickened line.

analyzed by computer (Larson & Messing, 1983) and by the facilities of the BIONET resource.

Derived Amino Acid Sequence. After the sequence of the 6-kb DNA fragment containing the *pur*L locus was determined, it was analyzed for open reading frames. Within the boundaries indicated by the complementation analysis, only one open reading frame capable of encoding a protein of greater than 100 000 daltons was found. This open reading frame extends from nucleotide 1294 to nucleotide 5178 (Figure 3) and encodes a protein of 141 418 daltons. The start of the *pur*L open reading frame was confirmed by comparison to the amino-terminal sequence of purified FGAM synthetase, obtained by automated Edman degradation of the purified protein:[2]

	M-E-I-L-(a)-G-(l)-P-A-L-(a)-A-F-R	60%
Edman degradation	A-M-E-I-L-(a)-G-(l)-P-A-L-(a)-A-F-R	40%
gene	M-M-E-I-L- R- G- S- P-A-L- S -A-F-R	

This open reading frame is preceded by a good ribosomal initiation site (Shine & Dalgarno, 1974), GAG, at nucleotides 1285–1287 (Figure 3).

An additional confirmation of the correctness of the FGAM synthetase sequence is indicated by the finding of nearly identical sequences corresponding to the glutamine binding domain of both chicken liver and *Salmonella* typhimurium FGAM synthetases. This region, nucleotides 4684–4710, is overlined in Figure 3.

Identification of purL Control Region. After identification of the *pur*L coding region, a 20-mer oligonucleotide designed to anneal to nucleotides 1324–1343 within the *pur*L coding region was synthesized. After annealing to total cellular RNA extracted from strain TX540 containing plasmid pJS80, primer extension with reverse transcriptase was carried out as described by Miller et al. (1986). The size of the primer extension product (Figure 4) indicated nucleotide 1248 as the most likely initiation point. Seven nucleotides upstream of the transcription initiation site, a consensus −10 region is found (Harley & Reynolds, 1987), and 15 nucleotides upstream of the −10 region, a good −35 region (four of six matches) is found (Raibaud & Schwartz, 1984).

Comparison of the *pur*L control region to the other *pur* loci control regions revealed a common region of dyad symmetry (nucleotides 1204–1216) (Smith & Daum, 1986; Tso et al., 1982). This sequence is located sufficiently close to the −35 region such that it may act as a binding site for a regulatory protein (the putative repressor protein) which controls transcription by altering binding of RNA polymerase.

Isolation of E. coli FGAM Synthetase. FGAM synthetase from *E. coli* strain TX635 containing pJS113 has been purified to homogeneity, Table I. From 1 g of cells, 23 mg of homogeneous protein was isolated in 60% overall yield.

[2] Two sequences occurred. The lower-case letters show less confidence. In fact, the first (a) round did contain R, and the (l) round contained serine.

EC_purL

```
        10          20          30          40          50
GATCCGTAAT GCCGAGGCTT TGCGCGGTAT TTTTGGTTAA CATCATCATG

        60          70          80          90         100
CCGCGCACAC CCGTCGGTGA AGTGGCCTGT GCATCCCAGT GCGATTCCTG

       110         120         130         140         150
ATAAGCAATA GCGGCCAGCA AACGCCAGTC AATTTCTTCG GCGTATTTCT

       160         170         180         190         200
CAAACAGGGG CTTTAACTGC GGCAGTACCG CATCGACGGC GCGTAAAAAT

       210         220         230         240         250
GTGCGCGTAT CGACGTAATC AAAATCATCG CCATGCCCCA GGTATTTCTC

       260         270         280         290         300
TTCAATGCGT GCCAGCGTAC CGTCTTCATT CATTTCGTTG AAGAAGTCGA

       310         320         330         340         350
GCAGGGCGGC GGAAAGGGTA TTATCGCCAT CTAACGGGCT AAACCAAGTC

       360         370         380         390         400
ACCGGTTGTT CATCGGTGAT ATCGAGCGCT ACGGCGAGCT CCGGGTGAAC

       410         420         430         440         450
GCGCTGAAAC AGGCTGATGG CGACAGAATC AGCAATGGTG TAATCGAGTT

       460         470         480         490         500
TTCCTTCGAT GACATCTTCC ATTAATTCCG CAGAGCCTTT TTTGTCGTCT

       510         520         530         540         550
ACCTTCCAGC TTAATTCCGG GAATTTTGTT TCTTTCAGGG TCTGGAGATC

       560         570         580         590         600
GTTAACCACC ACATGACCCG GTGCAACGGT GAGTCTGTCC GCCGTCAGGT

       610         620         630         640         650
TGCCCAGCGT ACGTGGGCGA TACTGACCCA CTTTATAAAC CAGTTGTTGT

       660         670         680         690         700
GACACGGAAT AATAGGTAGG GCCAGGCTGA TAATTTTTTA CCCGCTCACT

       710         720         730         740         750
GTTATAGACA AGTCCTGCCG CCAGCAGGTC GGCGTTACCA TTATCAAGGT

       760         770         780         790         800
CGTCAAACAG CTGGCTGATA TTCTGCCGCA CGGTCACTTT CAGTTTTACG

       810         820         830         840         850
CCGAGGTAAT CGGCAAACTG TTTCGCCAGT TCGTAATCCA GGCCAAAAGG

       860         870         880         890         900
TTTCCCGTTG ATTTCGTTAT AAGTCAGGGG AGTATGAATG GTGCTCACAC

       910         920         930         940         950
GCAACTCTCC CCGCGCTTGA ATGGCGGCGA TACGGTTGTC GGCTTTACCA

       960         970         980         990        1000
AACCAGGGAA TGGATGGCCA GAGAGCGACC GCGAGCAGCA GTGCCAGAAT

      1010        1020        1030        1040        1050
GCCGATGAAC AGATAATTAA TCTTTAATTT TTTCAATTAG TTAATTCTCT

      1060        1070        1080        1090        1100
GTGTCGTGCG CGTCCCAGCT TGAAAAAACG TAATAATAGT GAAAGGTTTA

      1110        1120        1130        1140        1150
CTCATAAATG AGCGGCATTT TGCGTAAACC TGCGCCAGAT GGCAACTTAT

      1160        1170        1180        1190        1200
TACAGCCATT GGCGGCACGC GTTGCTAATT CACGATGGTG ATTTTATTTC

      1210        1220        1230        1240        1250
CACGCAAACG GTTTCGTCAG CGCATCAGAT TCTTTATAAT GACGCCCGTT
   ============== -35                   -10          *

      1260        1270        1280        1290
TCCCCCCCTT GGGTACACCG AAAGCTTAGA AGACGAGAGA CTT ATG ATG
                                        S/D     MET MET

      1308                1323                1338
GAA ATT CTG CGT GGT TCG CCT GCA CTG TCG GCA TTC CGA ATC
Glu Ile Leu Arg Gly Ser Pro Ala Leu Ser Ala Phe Arg Ile

           1353                1368                1383
AAC AAA CTG CTG GCA CGT TTT CAG GCT GCC AGG CTC CCG GTT
Asn Lys Leu Leu Ala Arg Phe Gln Ala Ala Arg Leu Pro Val

                1398                1413
CAC AAT ATT TAC GCC GAG TAT GTC CAT TTT GCT GAC CTC AAT
His Asn Ile Tyr Ala Glu Tyr Val His Phe Ala Asp Leu Asn

1428                1443                1458
GCG CCG TTA AAC GAT GAT GAG CAC GCA CAA CTT GAA CGC CTG
Ala Pro Leu Asn Asp Asp Glu His Ala Gln Leu Glu Arg Leu

   1473                1488                1503
CTG AAA TAT GGC CCG GCA CTC GCC AGC CAC GCC CCG CAA GGC
Leu Lys Tyr Gly Pro Ala Leu Ala Ser His Ala Pro Gln Gly

       1518                1533                1548
AAA CTC CTG CTG GTG ACC CCG CGT CCT GGC ACC ATC TCT CCC
Lys Leu Leu Leu Val Thr Pro Arg Pro Gly Thr Ile Ser Pro

           1563                1578                1593
TGG TCT TCG AAA GCG ACC GAT ATT GCC CAT AAC TGC GGG CTA
Trp Ser Ser Lys Ala Thr Asp Ile Ala His Asn Cys Gly Leu

                1608                1623
CAA CAG GTA AAC CGC CTT GAG CGC GGC GTT GCT TAC TAT ATA
Gln Gln Val Asn Arg Leu Glu Arg Gly Val Ala Tyr Tyr Ile

1638                1653                1668
GAA GCC GGT ACG CTG ACC AAT GAA CAA TGG CAG CAG GTT ACC
Glu Ala Gly Thr Leu Thr Asn Glu Gln Trp Gln Gln Val Thr

   1683                1698                1713
GCT GAA CTG CAC GAC CGC ATG ATG GAA ACG GTC TTT TTT GCT
Ala Glu Leu His Asp Arg MET MET Glu Thr Val Phe Phe Ala

       1728                1743                1758
TTA GAT GAT GCA GAG CAG TTG TTT GCC CAC CAT CAA CCG ACT
Leu Asp Aep Ala Glu Gln Leu Phe Ala His His Gln Pro Thr

           1773                1788                1803
CCG GTT ACC AGC GTT GAT TTG CTG GGG CAG GGC CGT CAG GCG
Pro Val Thr Ser Val Asp Leu Leu Gly Gln Gly Arg Gln Ala

                1818                1833
CTG ATC GAC GCT AAC CTG CGT CTT GGC TTG GCT CTG GCG GAA
Leu Ile Asp Ala Asn Leu Arg Leu Gly Leu Ala Leu Ala Glu

1848                1863                1878
GAT GAA ATT GAC TAT CTG CAG GAT GCT TTC ACA AAG CTT GGT
Asp Glu Ile Asp Tyr Leu Gln Asp Ala Phe Thr Lys Leu Gly

   1893                1908                1923
CGT AAC CCG AAC GAC ATC GAA CTG TAT ATG TTT GCC CAG GCG
Arg Asn Pro Asn Asp Ile Glu Leu Tyr MET Phe Ala Gln Ala

       1938                1953                1968
AAC TCC GAG CAC TGC CGC CAC AAA ATT TTT AAC GCC GAC TGG
Asn Ser Glu His Cys Arg His Lys Ile Phe Asn Ala Asp Trp

           1983                1998                2013
GTT ATC GAT GGT GAA CAG CAG CCG AAA TCG CTG TTC AAG ATG
Val Ile Asp Gly Glu Gln Gln Pro Lys Ser Leu Phe Lys MET

                2028                2043
ATC AAA AAT ACT TTC GAA ACC ACG CCA GAT CAC GTT CTC TCT
Ile Lys Asn Thr Phe Glu Thr Thr Pro Asp His Val Leu Ser

2058                2073                2088
GCT TAT AAA GAT AAC GCC GCC GTA ATG GAA GGT TCT GAA GTG
Ala Tyr Lys Asp Asn Ala Ala Val MET Glu Gly Ser Glu Val

   2103                2118                2133
GGC CGC TAC TTT GCT GAC CAC GAA ACG GGC CGC TAC GAT TTC
Gly Arg Tyr Phe Ala Asp His Glu Thr Gly Arg Tyr Asp Phe

       2148                2163                2178
CAT CAG GAA CCG GCG CAT ATT CTG ATG AAA GTC GAA ACT CAC
His Gln Glu Pro Ala His Ile Leu MET Lys Val Glu Thr His

           2193                2208                2223
AAC CAC CCG ACG GCG ATT TCT CCG TGG CCG GGC GCG GCG ACC
Asn His Pro Thr Ala Ile Ser Pro Trp Pro Gly Ala Ala Thr

                2238                2253
GGT TCC GGC GGT GAA ATC CGC GAT GAA GGT GCC ACC GGG CGC
Gly Ser Gly Gly Glu Ile Arg Asp Glu Gly Ala Thr Gly Arg

2268                2283                2298
GGC GCA AAG CCG AAA GCG GGT CTG GTT GGT TTC TCC GTT TCC
Gly Ala Lys Pro Lys Ala Gly Leu Val Gly Phe Ser Val Ser

   2313                2328                2343
AAC CTG CGA ATT CCT GGC TTC GAA CAG CCG TGG GAA GAA GAT
Asn Leu Arg Ile Pro Gly Phe Glu Gln Pro Trp Glu Glu Asp

       2358                2373                2388
TTC GGT AAG CCT GAG CGC ATT GTC ACC GCG CTG GAC ATC ATG
Phe Gly Lys Pro Glu Arg Ile Val Thr Ala Leu Asp Ile MET

           2403                2418                2433
ACC GAA GGC CCG CTG GGC GGC GCG GCG TTT AAC AAC GAA TTT
Thr Glu Gly Pro Leu Gly Gly Ala Ala Phe Asn Asn Glu Phe

                2448                2463
GGT CGT CCG GCA CTG AAC GGC TAC TTC CGT ACT TAT GAA GAA
Gly Arg Pro Ala Leu Asn Gly Tyr Phe Arg Thr Tyr Glu Glu

2478                2493                2508
AAA GTG AAC AGC CAC AAC GGC GAA GAG CTG CGC GGT TAT CAC
Lys Val Asn Ser His Asn Gly Glu Glu Leu Arg Gly Tyr His

   2523                2538                2553
AAA CCG ATC ATG CTG GCG GGC GGG ATC GGC AAC ATT CGC GCC
Lys Pro Ile MET Leu Ala Gly Gly Ile Gly Asn Ile Arg Ala

       2568                2583                2598
GAT CAC GTA CAA AAA GGC GAG ATC AAC GTC GGT GCG AAG CTG
Asp His Val Gln Lys Gly Glu Ile Asn Val Gly Ala Lys Leu

           2613                2628                2643
GTC GTT CTC GGC GGC CCG GCA ATG AAC ATC GGT CTT GGC GGT
Val Val Leu Gly Gly Pro Ala MET Asn Ile Gly Leu Gly Gly

                2658                2673
GGT GCA GCG TCT TCT ATG GCG TCT GGT CAG TCT GAT GCC GAC
Gly Ala Ala Ser Ser MET Ala Ser Gly Gln Ser Asp Ala Asp
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2688                2703                2718
CTC GAC TTT GCT TCC GTA CAG CGC GAC AAC CCG GAG ATG GAG
Leu Asp Phe Ala Ser Val Gln Arg Asp Asn Pro Glu MET Glu

   2733                2748                2763
CGT CGC TGC CAG GAA GTG ATC GAC CGT TGC TGG CAG CTT GGT
Arg Arg Cys Gln Glu Val Ile Asp Arg Cys Trp Gln Leu Gly

         2778                2793                2808
GAT GCC AAC CCA ATC CTG TTT ATC CAC GAC GTT GGC GCT GGC
Asp Ala Asn Pro Ile Leu Phe Ile His Asp Val Gly Ala Gly

               2823                2838                2853
GGT CTT TCT AAC GCC ATG CCG GAA CTG GTG AGC GAC GGC GGG
Gly Leu Ser Asn Ala MET Pro Glu Leu Val Ser Asp Gly Gly

                  2868                2883
CGC GGC GGT AAA TTT GAA CTG CGC GAG ATT CTA AGC GAC GAA
Arg Gly Gly Lys Phe Glu Leu Arg Glu Ile Leu Ser Asp Glu

2898                2913                2928
CCG GGC ATG AGC CCG CTG GAA ATC TGG TGT AAC GAA TCC CAG
Pro Gly MET Ser Pro Leu Glu Ile Trp Cys Asn Glu Ser Gln

   2943                2958                2973
GAA CGC TAC GTG CTG GCG GTT GCT GCC GAT CAA TTA CCG CTG
Glu Arg Tyr Val Leu Ala Val Ala Ala Asp Gln Leu Pro Leu

         2988                3003                3018
TTT GAC GAA CTG TGT AAG CGT GAG CGC GCA CCC TAC GCG GTG
Phe Asp Glu Leu Cys Lys Arg Glu Arg Ala Pro Tyr Ala Val

               3033                3048                3063
ATT GGT GAA GCG ACC GAA GAA CTG CAT CTT TCT CTG CAC GAT
Ile Gly Glu Ala Thr Glu Glu Leu His Leu Ser Leu His Asp

                  3078                3093
CGT CAT TTT GAT AAT CAG CCG ATC GAT CTG CCG CTG GAC GTC
Arg His Phe Asp Asn Gln Pro Ile Asp Leu Pro Leu Asp Val

3108                3123                3138
CTG CTT GGT AAA ACG CCG AAG ATG ACC CGC GAT GTA CAA ACG
Leu Leu Gly Lys Thr Pro Lys MET Thr Arg Asp Val Gln Thr

   3153                3168                3183
CTG AAA GCG AAA GGC GAC GCG CTG GCC CGT GAA GGG ATC ACC
Leu Lys Ala Lys Gly Asp Ala Leu Ala Arg Glu Gly Ile Thr

         3198                3213                3228
ATT GCA GAC GCG GTG AAA CGT GTG CTG CAT CTG CCG ACT GTG
Ile Ala Asp Ala Val Lys Arg Val Leu His Leu Pro Thr Val

               3243                3258                3273
GCG GAA AAA ACC TTC CTG GTG ACC ATT GGC GAC CGC AGC GTA
Ala Glu Lys Thr Phe Leu Val Thr Ile Gly Asp Arg Ser Val

                  3288                3303
ACC GGC ATG GTA GCG CGC GAT CAG ATG GTG GGG CCG TGG CAG
Thr Gly MET Val Ala Arg Asp Gln MET Val Gly Pro Trp Gln

3318                3333                3348
GTG CCG GTC GCT AAC TGC GCG GTC ACT ACC GCC AGC CTC GAC
Val Pro Val Ala Asn Cys Ala Val Thr Thr Ala Ser Leu Asp

   3363                3378                3393
AGC TAC TAC GGT GAA GCG ATG GCG ATT GGC GAG CGT GCG CCG
Ser Tyr Tyr Gly Glu Ala MET Ala Ile Gly Glu Arg Ala Pro

         3408                3423                3438
GTT GCG CTG CTG GAT TTC GCC GCC TCT GCC CGT CTG GCG GTC
Val Ala Leu Leu Asp Phe Ala Ala Ser Ala Arg Leu Ala Val

               3453                3468                3483
GGT GAA GCG TTA ACC AAC ATC GCC GCA ACA CAA ATT GGC GAT
Gly Glu Ala Leu Thr Asn Ile Ala Ala Thr Gln Ile Gly Asp

                  3498                3513
ATC AAA CGC ATC AAA CTT TCC GCC AAC TGG ATG GCG GCG GCA
Ile Lys Arg Ile Lys Leu Ser Ala Asn Trp MET Ala Ala Ala

3528                3543                3558
GGC CAC CCT GGT GAA GAT GCG GGC CTG TAT GAA GCC GTT AAA
Gly His Pro Gly Glu Asp Ala Gly Leu Tyr Glu Ala Val Lys

   3573                3588                3603
GCC GTG GGC GAA GAG CTT TGT CCG GCG CTG GGC CTG ACG ATC
Ala Val Gly Glu Glu Leu Cys Pro Ala Leu Gly Leu Thr Ile

         3618                3633                3648
CCG GTG GGT AAA GAC TCC ATG TCG ATG AAA ACC CGC TGG CAG
Pro Val Gly Lys Asp Ser MET Ser MET Lys Thr Arg Trp Gln

               3663                3678                3693
GAA GGT AAC GAA GAG CGC GAA ATG ACG TCG CCG CTG TCG CTG
Glu Gly Asn Glu Glu Arg Glu MET Thr Ser Pro Leu Ser Leu

                  3708                3723
GTG ATT TCT GCA TTT GCC CGC GTG GAA GAT GTA CGT CAC ACC
Val Ile Ser Ala Phe Ala Arg Val Glu Asp Val Arg His Thr

3738                3753                3768
ATC ACG CCG CAG CTT TCT ACC GAA GAT AAC GCA CTG CTG CTG
Ile Thr Pro Gln Leu Ser Thr Glu Asp Asn Ala Leu Leu Leu

   3783                3798                3813
ATT GAT TTG GGC AAA GGC AAT AAC GCG CTG GGC GCA ACG GCG
Ile Asp Leu Gly Lys Gly Asn Asn Ala Leu Gly Ala Thr Ala

         3828                3843                3858
CTG GCG CAG GTT TAT CGT CAG CTT GGC GAC AAA CCG GCA GAT
Leu Ala Gln Val Tyr Arg Gln Leu Gly Asp Lys Pro Ala Asp

               3873                3888                3903
GTA CGC GAT GTC GCG CAA CTG AAA GGC TTC TAT GAC GCG ATT
Val Arg Asp Val Ala Gln Leu Lys Gly Phe Tyr Asp Ala Ile

                  3918                3933
CAG GCG CTG GTT GCA CAG CGT AAG CTG CTG GCG TAT CAC GAC
Gln Ala Leu Val Ala Gln Arg Lys Leu Leu Ala Tyr His Asp

3948                3963                3978
CGC TCT GAT GGC GGC CTG CTG GTA ACG CTG GCG GAA ATG GCC
Arg Ser Asp Gly Gly Leu Leu Val Thr Leu Ala Glu MET Ala

   3993                4008                4023
TTT GCT GGT CAT TGT GGC ATT GAC GCG GAT ATC GCC ACT CTG
Phe Ala Gly His Cys Gly Ile Asp Ala Asp Ile Ala Thr Leu

         4038                4053                4068
GGT GAC GAT CGC CTG GCG GCG TTG TTT AAC GAA GAA CTG GGT
Gly Asp Asp Arg Leu Ala Ala Leu Phe Asn Glu Glu Leu Gly

               4083                4098                4113
GCG GTG ATT CAG GTT CGT GCC GCT GAC CGT GAA GCG GTC GAG
Ala Val Ile Gln Val Arg Ala Ala Asp Arg Glu Ala Val Glu

                  4128                4143
TCC GTA CTG GCA CAG CAT GGG CTT GCT GAT TGT GTC CAT TAT
Ser Val Leu Ala Gln His Gly Leu Ala Asp Cys Val His Tyr

4158                4173                4188
GTA GGG CAG GCG GTT TCC GGT GAC CGT TTT GTG ATT ACC GCC
Val Gly Gln Ala Val Ser Gly Asp Arg Phe Val Ile Thr Ala

   4203                4218                4233
AAC GGG CAG ACT GTA TTC AGC GAA AGC CGC ACC ACG TTG CGT
Asn Gly Gln Thr Val Phe Ser Glu Ser Arg Thr Thr Leu Arg

         4248                4263                4278
GTC TGG TGG GCA GAA ACT ACC TGG CAG ATG CAG CGC CTG CGT
Val Trp Trp Ala Glu Thr Thr Trp Gln MET Gln Arg Leu Arg

               4293                4308                4323
GAC AAC CCG GAG TGT GCC GAT CAG GAG CAT CAG GCG AAA TCT
Asp Asn Pro Glu Cys Ala Asp Gln Glu His Gln Ala Lys Ser

                  4338                4353
AAC GAC GCC GAT CCG GGC CTG AAT GTA AAA CTG TCG TTC GAT
Asn Asp Ala Asp Pro Gly Leu Asn Val Lys Leu Ser Phe Asp

4368                4383                4398
ATC AAC GAA GAT GTG GCA GCA CCG TAT ATT GCC ACT GGC GCA
Ile Asn Glu Asp Val Ala Ala Pro Tyr Ile Ala Thr Gly Ala

   4413                4428                4443
CGT CCG AAA GTT GCC GTA CTG CGT GAG CAG GGC GTG AAC TCG
Arg Pro Lys Val Ala Val Leu Arg Glu Gln Gly Val Asn Ser

         4458                4473                4488
CAT GTT GAA ATG GCG GCA GCT TTC CAC CGT GCA GGC TTT GAT
His Val Glu MET Ala Ala Ala Phe His Arg Ala Gly Phe Asp

               4503                4518                4533
GCT ATC GAC GTG CAT ATG AGT GAC CTG CTG ACC GGA CGC ACG
Ala Ile Asp Val His MET Ser Asp Leu Leu Thr Gly Arg Thr

                  4548                4563
GGC CTG GAA GAT TTC CAC GCC CTG GTC GCG TGC GGT GGT TTC
Gly Leu Glu Asp Phe His Ala Leu Val Ala Cys Gly Gly Phe

4578                4593                4608
TCC TAC GGT GAT GTG CTG GGT GCC GGT GAA GGT TGG GCG AAG
Ser Tyr Gly Asp Val Leu Gly Ala Gly Glu Gly Trp Ala Lys

   4623                4638                4653
TCA ATC CTG TTC AAT GAC CGT GTA CGC GAT GAG TTT GCA ACC
Ser Ile Leu Phe Asn Asp Arg Val Arg Asp Glu Phe Ala Thr

         4668                4683                4698
TTC TTC CAC CGT CCG CAA ACG CTG GCG CTG GGG GTA TGT AAC
Phe Phe His Arg Pro Gln Thr Leu Ala Leu Gly Val Cys Asn

               4713                4728                4743
GGT TGC CAG ATG ATG TCT AAT CTG CGT GAA CTG ATC CCA GGT
Gly Cys Gln MET MET Ser Asn Leu Arg Glu Leu Ile Pro Gly

                  4758                4773
AGT GAG TTG TGG CCA CGT TTT GTG CGC AAT ACC TCC GAT CGC
Ser Glu Leu Trp Pro Arg Phe Val Arg Asn Thr Ser Asp Arg

4788                4803                4818
TTT GAA GCG CGT TTC AGC CTG GTT GAA GTA ACC CAA AGC CCG
Phe Glu Ala Arg Phe Ser Leu Val Glu Val Thr Gln Ser Pro

   4833                4848                4863
TCT CTG CTG TTG CAG GGG ATG GTG GGC TCG CAA ATG CCG ATT
Ser Leu Leu Leu Gln Gly MET Val Gly Ser Gln MET Pro Ile

         4878                4893                4908
GCT GTC TCT CAT GGT GAA GGG CGC GTG GAA GTG CGT GAT GCG
Ala Val Ser His Gly Glu Gly Arg Val Glu Val Arg Asp Ala

               4923                4938                4953
GCG CAT CTG GCG GCA CTG GAA AGC AAA GGG CTG GTG GCA CTG
Ala His Leu Ala Ala Leu Glu Ser Lys Gly Leu Val Ala Leu
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                4968                    4983
CGC TAT GTC GAT AAC TTC GGC AAA GTC ACT GAA ACC TAC CCG
Arg Tyr Val Asp Asn Phe Gly Lys Val Thr Glu Thr Tyr Pro

4998                5013                    5028
GCT AAC CCG AAC GGT TCA CCG AAC GGT ATT ACG GCA GTC ACG
Ala Asn Pro Asn Gly Ser Pro Asn Gly Ile Thr Ala Val Thr

   5043                    5058                    5073
ACT GAA AGT GGT CGA GTC ACC ATT ATG ATG CCG CAC CCG GAA
Thr Glu Ser Gly Arg Val Thr Ile MET MET Pro His Pro Glu

        5088                    5103                    5118
CGT GTT TTC CGT ACT GTC AGC AAC TCC TGG CAT CCG GAA AAC
Arg Val Phe Arg Thr Val Ser Asn Ser Trp His Pro Glu Asn

            5133                    5148                    5163
TGG GGC GAG GAT GGC CCA TGG ATG CGC ATT TTC CGC AAT GCG
Trp Gly Glu Asp Gly Pro Trp MET Arg Ile Phe Arg Asn Ala

                5178            5191        5201        5211
CGT AAG CAG TTG GGG TAA GTCGTCAGCC CATTGGTTTT GCAGCCCGGA
Arg Lys Gln Leu Gly  .

      5221        5231        5241        5251        5261
TGCGGCATTA AGCACCAACC ACTATCATAA GCTCCCAACT CCGGGAGCTT

      5271        5281        5291        5301        5311
TTTTGTGTCT GTAAATCACG ACAATGGGTG GTTTGCCGTG TCGCTTTCTG

      5321        5331        5341        5351        5361
GCGACACTTA ACTCATTGAT TTTAATATTA TCTAATAAGT TTATCTTAAG
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      5371        5381        5391        5401        5411
TTGTCTCTTT TTAGCGACAC AGTGGCTGAT TTACATTCAA ACTGCGGAGG

      5421        5431        5441        5451        5461
AGACATAAAC CCCTCAAAGC AAGCTATAAT CGATAGCTTA CCAAACTATT

      5471        5481        5491        5501        5511
TTCTTTATTG GCACAGTTAC TGCATAATAG TAACCAGTGG CTCATTCACC

      5521        5531        5541        5551        5561
GACTTATGTC AGCCCCTTCG GGACGTGCTA CATAAAATAC GAATGACGCA

      5571        5581        5591        5601        5611
CAACAAGGTG CCTGCCGTCC AACTTCTGAT ATCAGCGTAG CTATATCAAC

      5621        5631        5641        5651        5661
CATCGGGCGA AACGTCGAGT TAGGCACCGC CTTATTCCAT AACAAAGCCG

      5671        5681        5691        5701        5711
GGTAATTCCC GGCTTTGTTG TATCTGAACT TCCCCTCGGT TAGCATCAGG

      5721        5731        5741        5751        5761
CTATTCGCGT CTGACGAGAG TAACACCTTG AAACGCTGGC CCGTTTTTCC

      5771        5781        5791        5801        5811
CCGCTCATTA CGACAACTGG TAATGCTGGC ATTTTTGCTG ATTCTGCTGC

      5821        5831        5841        5851        5861
CCCTGTTGGT GCTGGCATGG CAAGCCTGGC AAAGCCTGAA TGCGCTTAGC

5865
GATC
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FIGURE 3: Nucleotide and deduced amino acid sequence of *pur*L. The DNA sequence of the sense strand of the *pur*L gene is shown. It is numbered from a *Sau*3A site as the 5′ end and includes 1293 nucleotides of upstream DNA, 3885 nucleotides of coding region, and 687 nucleotides beyond the coding region. The proposed *pur*L Shine–Dalgarno sequence (Shine & Dalgarno, 1974) at nucleotides 1285–1287 is indicated as S/D. The amino acid residues confirmed by sequencing are overlined. The FGAM synthetase sequence matching the active site sequence of the *Salmonella* and chicken liver FGAM synthetase is also overlined. At 58 bp downstream from the *pur*L structural gene is a region of dyad symmetry (nucleotides 5240–5261) with the characteristics of a ρ independent terminator (Holmes et al., 1983), a ΔG of −18.8 kcal/mol (Tinoco et al., 1973), and that could represent the termination site of *pur*L transcription. Nucleotide 1248, identified by primer extension studies, is marked with an asterisk. The inferred −10 and −35 regions are underlined and labeled. The common sequence (nucleotides 1204–1216) shared with the other *pur* loci is also underlined.

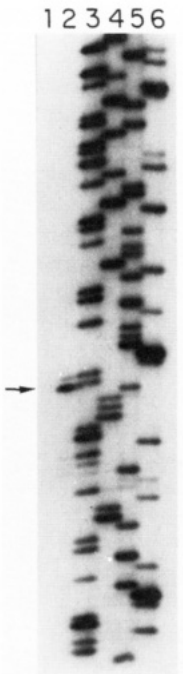

FIGURE 4: Primer extension analysis of the 5′ end of *pur*L mRNA. An oligonucleotide complementary to nucleotides 1324–1343 within the *pur*L coding region was hybridized to total cellular RNA from strain TX540 containing plasmid pJS80. It was extended with reverse transcriptase, and the products were analyzed on a sequencing gel. Lane 1 is a control reaction lacking the 20-mer oligonucleotide, lane 2 is the primer extension product, and lanes 3–6 are the dideoxy sequencing reactions used as size standards. The major primer extension product is 96 nucleotides in length.

Table I: Purification of FGAM Synthetase

step[a]	vol (mL)	protein (mg/mL)	total act. (units)	sp act. (units mg^{-1})
crude	5.2	14.4	81	1.08
40–55% $(NH_4)_2SO_4$	0.83	57.0	59	1.25
Sephadex G-25	31	1.4	64	1.47
DEAE-52	145	0.16	50	2.15

[a] 1.1 g of cell paste.

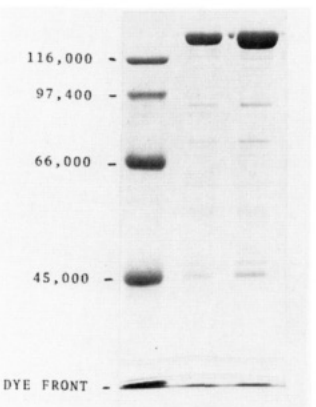

FIGURE 5: SDS gel (7.5% acrylamide) of *E. coli* FGAM synthetase: lane 1, molecular weight standards; lane 2, 20 μg of FGAM synthetase from the DEAE-52 column (Table I); lane 3, 40 μg.

Physical Characterization of FGAM Synthetase. SDS gel electrophoresis (Figure 5) and Sephadex G-150 chromatography of *E. coli* FGAM synthetase both indicate by comparison with standards of known molecular weight that FGAM synthetase is a single polypeptide of M_r = 135K and 125K, respectively. These numbers are similar to the previously determined molecular weight of the chicken liver (133K) and *Salmonella* (135K) protein and are slightly less than the M_r = 141K determined by the *E. coli* gene sequence.

Catalytic Properties of FGAM Synthetase. E. coli FGAM synthetase has been shown to catalyze the stoichiometric conversion of FGAR, MgATP, and glutamine to FGAM, MgADP, and glutamate with the assay procedures described under Materials and Methods.

These studies are a prerequisite to the investigation of whether *E. coli* FGAM synthetase, in analogy with all other amidotransferase thus far examined, exhibits glutaminase

Table II: Comparison of Conserved Regions of Glutamine Amide Transfer[a]

Enzyme (Source)		ref.
FGAMS (E. Coli)	NH_2--1120---T L A L G V C N G C Q M M---121---S N S W H P E---20---CO_2H	1
FGAMS (S. typhimurium)	A L G V C	CO_2H 2
FGAMS (B. subtilis)	NH_2---79----K P V L G V C N G F Q I L---97----G M M P H P E---31---CO_2H	3
CTPS (E. Coli)	NH_2---72----I P Y L G I C L G M Q V A---125---A C Q F H P E---28---CO_2H	4
CPS (E. Coli)	NH_2---263---I P V F G I C L G H Q L L---73----S F Q G H P E---27---CO_2H	5
PABS (E. Coli)	NH_2---72----L P I L G V C L G H Q A M---78----G V Q F H P E---17---CO_2H	6
ASII (E. Coli)	NH_2---80----L P I I G I C L G H Q A I---75----G F Q F H P E---33---CO_2H	7
GMPS (E. Coli)	NH_2---79----V P V F G V C Y G M Q T M---84----G V Q F H P E---23---CO_2H	8

[a] References: (1) this paper; (2) Dawid et al., 1963; (3) Ebbole & Zalkin, 1987; (4) Weng et al., 1986; (5) Piette et al., 1984; (6) Kaplan et al., 1985; (7) Nichols et al., 1980; (8) Zalkin et al., 1985.

activity and the ability to use NH_3 in place of glutamine. Incubation of glutamine with *E. coli* FGAM synthetase at pH 7.3 (optimum) resulted in the production of glutamate at 0.02% the rate of the normal reaction. This result contrasts with previously reported glutaminase activities of 0.5% for the chicken liver (Mizobuchi & Buchanan, 1968a) and 0.6% for the Ehrlich ascites tumor cell enzyme (Chu & Henderson, 1972). A number of glutamine-requiring enzymes exhibit enhanced glutaminase activity in the presence of one of their additional substrates, or in the presence of substrate analogues. The glutaminase activity of FGAM synthetase was therefore determined in the presence of FGAR, MgATP, or FGAR and MgAMP-PCP. The glutaminase activity was unaffected by any of these alternative conditions. Previous studies by Mizobuchi and Buchanan (1968a) showed that incubation of the chicken liver enzyme with glutamine and NH_2OH resulted in the production of the γ-glutamylhydroxamate at a rate of 80% that of the conversion of FGAR to FGAM. A similar experiment using the *E. coli* enzyme failed to reveal production of any detectable hydroxamate. Thus the glutamate binding domains of the chicken liver and *E. coli* synthetases, even though their active site cysteines are situated in similar environments (Table II), possess marked differences in accessibility to small molecules such as H_2O and NH_2OH.

As in the case of other amidotransferases, *E. coli* FGAM synthetase is also capable of using ammonia in place of glutamine as the nitrogen donor in the reaction. However, the K_m for ammonium chloride is 1 M at pH 8.0 and the V_{max} is only 2% that of the glutamine reaction. These results again contrast with those reported for the chicken liver synthetase by Mizobuchi and Buchanan (1968): K_m of 10 mM and a V_{max} of 5% the normal turnover number.

Characterization of the FGAM Synthetase–Glutamine Complex. Chicken liver FGAM synthetase (Mizobuchi & Buchanan, 1968a), *E. coli* CTP synthetase (Levitzki & Koshland, 1971), and *E. coli* carbamoylphosphate synthetase (Wellner et al., 1973) have in common the ability to form an isolable 1:1 complex with glutamine. This subgroup of amidotransferases has a homologous *trp*G-derived glutamine amide transfer domain containing both an invariant and catalytically essential cysteine and imidazole (Table II) (Ebbole & Zalkin, 1987). The nature of the complex formed by CTP synthetase and chicken liver FGAM synthetase was proposed to consist of a covalent γ-glutamyl enzyme thioester. The complex formed by carbamoylphosphate synthetase was proposed to consist of a tightly bound glutamine (Wellner et al., 1973; Rubino et al., 1986). A similar series of experiments were undertaken to determine the composition of a complex, if formed, between *E. coli* FGAM synthetase and glutamine. Incubation of [U-^{14}C]-L-glutamine with *E. coli* FGAM synthetase for 5 min, followed by chromatography on Sephadex G-50, resulted in coelution of radioactivity with the protein. A control experiment in which [U-^{14}C]glutamate replaced glutamine indicated no coelution of radioactivity with protein.

The half-life for the complex is 22 min at 4 °C, determined from a semilog plot of the binding ratio (glutamine:enzyme) as a function of time. Extrapolation to zero time gives a binding ratio of 0.96. Thus FGAM synthetase from *E. coli* is also capable of binding 1 equiv of glutamine per monomer.

The nature of this complex was investigated by determining the composition of the radioactive material which coeluted with the enzyme. When the isolated complex was allowed to stand at 4 °C for 4 h or was denatured by the addition of 20% trichloroacetic acid, the radioactivity was all found to comigrate with glutamate on an anion-exchange column. A control experiment in which glutamine was treated in an identical manner gave no detectable hydrolysis to glutamate. This result suggested that the glutamine may be bound as a thiohemiaminal or as a thioester in the complex and that the intermediate(s) can hydrolyze to form glutamate. The slow breakdown of the complex to glutamate is consistent with the slow rate of glutaminase activity catalyzed by the enzyme. To determine if glutamine is covalently bound as a thioester to FGAM synthetase, the isolated complex was treated with hydroxylamine at pH 7.2 for 20 min. Hydroxylamine is known to react quantitatively with thioesters to form hydroxamic acids (Stadtman, 1957). As shown in Table III, no glutamylhydroxamic acid was detectable, and 95% of the radioactivity was recovered as glutamate. Inaccessibility of hydroxylamine to the active site provides a possible explanation of these observations.

In order to enhance the accessibility of hydroxylamine to the putative thioester, the hydroxylamine trapping experiment

Table III: Trapping of Enzyme–Glutamine Complex with Hydroxylamine

complex treatment	enzyme (nmol)	complex (nmol)	glutamate (nmol)	glutamine or γ-glutamyl-hydroxamic acid (nmol)
1 M hydroxylamine	6.0	4.0	3.8	undetectable
1 M hydroxylamine with 6 M guanidine hydrochloride	5.0	3.4	0.45	2.8

was repeated in the presence of 6 M guanidine hydrochloride at pH 7.2. Isolation of the products from this experiment established that now 85% of the radioactivity comigrated with γ-glutamylhydroxamic acid, while 15% comigrated with glutamate on an anion-exchange column (Table III). Since glutamine and γ-glutamylhydroxamic acid coelute by this chromatographic procedure, the method of Levintow et al. (1955) was utilized to distinguish between these two alternatives. Previous investigators have established and we have substantiated that under a defined set of conditions that γ-glutamylhydroxamic acid can be quantitatively converted to pyrrolidonecarboxylic acid while only 4% of the glutamine and none of the glutamate treated in the same manner are converted to this product. Therefore, our sample was heated at 100 °C for 7.5 min at neutral pH and rechromatographed on an anion-exchange column. The eluents were then chromatographed on a cellulose F thin-layer plate (Materials and Methods) which has been shown to separate L-glutamate, L-glutamine, and pyrrolidonecarboxylic acid (Mizobuchi & Buchanan, 1968a). Location of the radioactive material by scintillation counting revealed that it all comigrated with pyrrolidonecarboxylic acid. These experiments show that, as in the case of the chicken liver FGAM synthetase (Mizobuchi & Buchanan, 1968a) and *E. coli* cytidine-5′-triphosphate synthetase (Levitzki & Koshland, 1971), the enzyme–glutamine complex consists of a covalent γ-glutamyl enzyme thioester or the tetrahedral precursor to the thiol ester which is capable of undergoing hydrolysis to glutamate.

Having established that the enzyme–glutamine complex consists of a γ-glutamyl enzyme thioester, a question remains concerning the location of the "putative ammonia equivalent". Several possibilities exist: the ammonia may be held tightly (covalently or noncovalently) at the active site of FGAM synthetase ready to undergo reaction when MgATP and FGAR are added, or it may have dissociated from the complex. To determine if the enzyme–glutamine complex is chemically competent to produce products, the following experiment was carried out. The enzyme–glutamine complex was isolated from a Sephadex G-50 column and immediately added to a reaction mixture containing FGAR, MgATP, and AIR synthetase. If any FGAM is produced, AIR synthetase will convert it to AIR which can be quantitated by the Bratton–Marshall assay. When 6.8 nmol of complex is treated in this fashion, no AIR is formed (Table IV). A control to set a lower limit on product detection was run in which glutamine (2.1 or 4.2 nmol) was included in an identical reaction mixture. The expected amount of AIR was produced (Table IV). This experiment demonstrates that the enzyme–glutamine complex is not chemically competent for product formation in the absence of added glutamine. Hence, at least 99% of the ammonia liberated by thioester formation either remains bound in a chemically incompetent fashion or more probably has dissociated from the active site. These observations seem to rule

Table IV: Turnover of Enzyme–Glutamine Complex To Form FGAM

substrates added to E·Gln complex	enzyme (nmol)	complex (nmol)	AIR (nmol)
MgATP, FGAR	10	6.8	undetectable
MgATP, FGAR, 2.1 nmol of Gln	7.3	4.8	2.0
MgATP, FGAR, 4.2 nmol of Gln	6.6	4.6	4.3

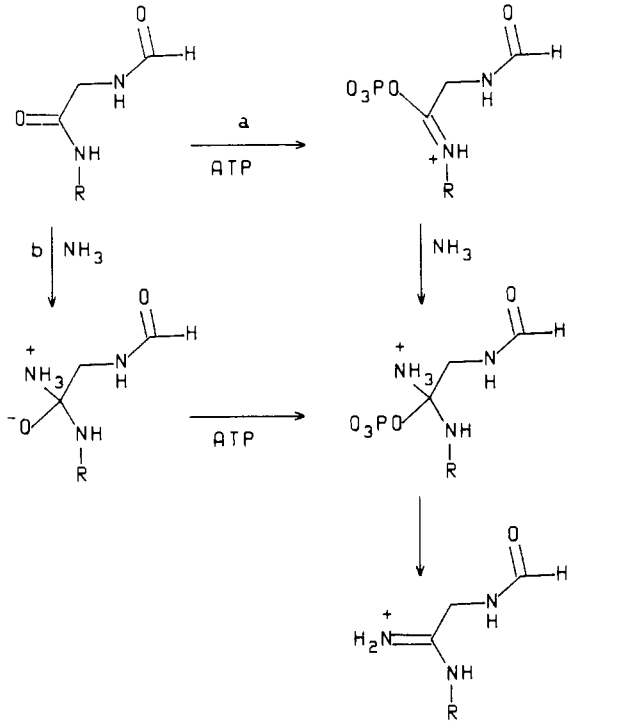

FIGURE 6: Proposed mechanism for the conversion of FGAR to FGAM catalyzed by FGAM synthetase. R = ribose 5-phosphate.

out the chemically attractive possibility that the putative ammonia is held covalently at the active site as a thiohemiaminal ready to deliver NH_3 on binding of FGAR and ATP.

Transfer of ^{18}O from [^{18}O]FGAR to P_i. Several mechanisms for the conversion of FGAR and FGAM have been proposed for the chicken liver FGAM synthetase (Schendel & Stubbe, 1986) (Figure 6). Pathway a (Figure 6) originally suggested by Satterthwait and Westheimer (1980) involves phosphorylation of the amide oxygen of FGAR by ATP to form a phosphorylated FGAR intermediate. This species is then attacked by putative NH_3 produced from glutamine to form a tetrahedral intermediate which collapses with subsequent loss of phosphate to form FGAM. Additional support for this type of mechanism comes from the recent work by von der Saal et al. (1985) investigating cytidine-5′-triphosphate synthetase using the positional isotope exchange methods. Pathway b, a modification of a mechanism proposed by Mizobuchi et al. (1968), involves attack on the carbonyl of the amide by ammonia to form a carbinolamine which is then phosphorylated by ATP, followed by loss of phosphate to give FGAM. A similar mechanism in which the attack by ammonia and phosphorylation by ATP occur in a concerted fashion is also possible. In addition or alternatively, a phosphorylated enzyme intermediate must be considered.

Both mechanisms in Figure 6 predict that the oxygen from the amide of FGAR would be transferred to the inorganic phosphate derived from ATP hydrolysis. To test this hypothesis, FGAR was prepared with 50% ^{18}O in the amide

Table V: Dead-End Inhibition of *E. coli* FGAM Synthetase

substrate	constant substrates	inhibitor	inhibition type	K_{is} (mM)	K_{ii} (mM)
Gln	FGAR (72 μM), ATP (106 μM)	glutamate	C	1.6 ± 0.16	
FGAR	Gln (88 μM), ATP (106 μM)	glutamate	NC	13 ± 1.8	52 ± 9.9
ATP	FGAR (72 μM), Gln (88 μM)	glutamate	NC	21 ± 5.3	16 ± 2.1
Gln	FGAR (89 μM), ATP (101 μM)	albizziin	C	8.9 ± 0.69	
FGAR	Gln (110 μM), ATP (139 μM)	albizziin	NC	15 ± 4.7	23 ± 6.2
ATP	FGAR (89 μM), Gln (110 μM)	albizziin	NC	13 ± 2.0	18 ± 1.6
ATP	FGAR (72 μM), Gln (138 μM)	AMP-PCP[a]	C	0.066 ± 0.005	
FGAR	ATP (134 μM), Gln (138 μM)	AMP-PCP	NC	2.0 ± 0.37	6.4 ± 1.8
Gln	FGAR (72 μM), ATP (134 μM)	AMP-PCP	UC		2.2 ± 0.10

[a] Adenosine 5′-(β,γ-methylenetriphosphate).

oxygen. This was accomplished by mixing equal amounts of [1-$^{16}O_2$,^{13}C]glycine and [1-$^{18}O_2$,^{13}C]glycine (Figure 7A) and biosynthetically incorporating the glycine into FGAR (Materials and Methods). The [$^{18}O/^{16}O$]FGAR is incubated with FGAM synthetase, MgATP, and glutamine, and the inorganic phosphate which is produced is isolated by DEAE-Sephadex anion-exchange chromatography. A ^{31}P NMR spectrum of the isolated inorganic phosphate was taken and is shown in Figure 7B. This spectrum indicates that a mixture of [^{16}O]P_i and [^{18}O]P_i is formed in a ratio which is identical with the $^{16}O/^{18}O$ ratio in the starting glycine. Therefore, the amide oxygen of FGAR is quantitatively transferred to the phosphate derived from ATP hydrolysis during the conversion of FGAR to FGAM.

Kinetic Studies. In order to determine the optimum conditions for kinetic analysis, the pH dependence and metal ion specificity of FGAM synthetase were investigated. The enzyme has a pH optimum of 7.2 with only 50% of this activity being retained at pH 6.2 or 8.0. In addition, 20 mM free Mg^{2+} and 10 mM free K^+ ions were found to be required for optimal activity. The requirement for high concentrations of free Mg^{2+} suggest a structural role for the metal in addition to its substrate role as MgATP. These results can be compared with results from the chicken liver enzyme which has a pH optimum of 8 and requires 60 mM free K^+ and 20 mM free Mg^{2+} for optimal activity (Mizobuchi & Buchanan, 1968b). Determination of the Michaelis constants for the three substrates resulted in a K_m = 64 μM for glutamine, a K_m = 51 μM for MgATP, and a K_m = 30 μM for FGAR. The K_m for MgATP is considerably different from the K_m of 1.5 mM reported for both the chicken liver and tumor cell enzymes, while the K_m for glutamine and FGAR are similar, 40 μM and 100 μM (chicken liver) and 110 μM and 110 μM (Erlich ascites tumor cells), respectively (Li & Buchanan, 1971; Chu & Henderson, 1972).

Initial Velocity and Product and Dead-End Inhibition Studies. Initial velocity and product and dead-end inhibition studies were undertaken to determine the order of addition of substrates in this three-substrate, four-product system. The initial velocity studies resulted in an intersecting pattern when FGAR and MgATP were varied at fixed levels of glutamine. However, when FGAR and glutamine were varied at fixed levels of MgATP, or when MgATP and glutamine were varied at fixed levels of FGAR, parallel patterns were obtained. These parallel initial velocity patterns suggest a ping-pong mechanism in which an irreversible step, presumably product release, occurs between the binding of glutamine and the binding of the other two substrates. The intersecting initial velocity pattern between FGAR and MgATP suggests a sequential mechanism for the binding of these two substrates. Two ping-pong mechanisms exist which are consistent with these initial velocity patterns: (1) A mechanism in which glutamine binds first followed by release of glutamate and then the addition of MgATP and FGAR followed by reaction to form products or (2) a mechanism in which MgATP and FGAR bind followed by an irreversible step, possibly the release of ADP, and then the addition of glutamine followed by reaction to form products. However, both mechanisms 1 and 2 are unappealing because of the absence of glut-

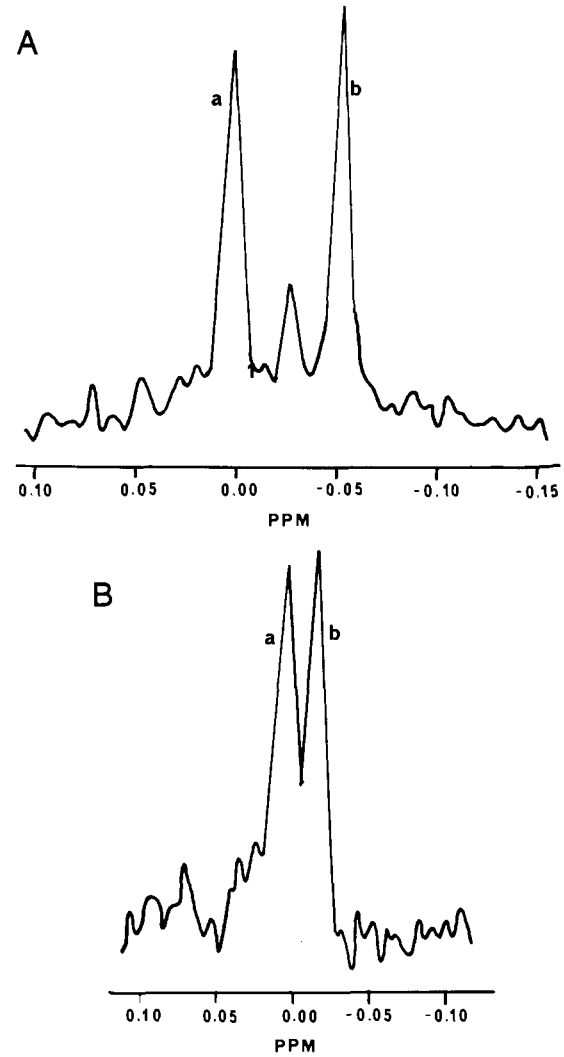

FIGURE 7: (A) A 50.3-MHz ^{13}C NMR spectrum of a mixture of [1-^{13}C,$^{18}O_2$]glycine and [1-^{13}C,$^{16}O_2$]glycine. Peak a is [1-^{13}C,$^{16}O_2$]glycine and peak b is [1-^{13}C,$^{18}O_2$]glycine. The peak in the center is due to [1-^{13}C,^{16}O,^{18}O]glycine. The spectrum was obtained from 100 acquisitions using broad-band proton decoupling with a 35° pulse angle, a 4K data block, a ±200-Hz sweep width, a 10-s acquisition time, and a 10-s pulse delay. A line-broadening factor of 0.2 Hz was applied to the FID before Fourier transformation. (B) An 80.9-MHz ^{31}P NMR spectrum showing the inorganic phosphate produced from the reaction of FGAM synthetase, MgATP, glutamine, and a 1:1 mixture of [^{18}O]FGAR and [^{16}O]FGAR. Peak a is [^{16}O]FGAR, and peak b is [^{18}O]FGAR.

amine/glutamate exchange (mechanism 1) and the slow rate of ATP/ADP exchange (mechanism 2).

To determine which, if either, of these ping-pong mechanisms pertains, product and dead-end inhibition studies were carried out. As shown in Table V, glutamate was found to be competitive with glutamine and noncompetitive with both FGAR and MgATP. This rules out mechanism 1 since this mechanism predicts that glutamate would show a competitive inhibition pattern with either FGAR or MgATP. When albizziin, a glutamine analogue, was used as an inhibitor, it was found to be competitive with glutamine and noncompetitive with both FGAR and MgATP. These data also suggest that the reaction does not proceed by mechanism 1 since this mechanism would predict that albizziin would be uncompetitive with both FGAR and MgATP. These inhibition studies and preliminary exchange studies have therefore caused us to reevaluate our interpretation of our initial velocity studies. Since parallel initial velocity patterns are often difficult to distinguish from patterns which intersect at some point a great distance from the y axis, alternative mechanisms were sought by computer fitting of the initial velocity data to eq 3 and by graphical analysis methods described by Viola and Cleland (1982). From these analyses, the terms corresponding to coeff B, coeff C, and K_b in eq 3 were found to be undefined. Therefore, the initial velocity data were refit to a form of eq 3 which had these three terms removed. This form of the equation gave the best fit to the initial velocity data and corresponds to the ordered mechanisms shown in Figure 8 (Viola & Cleland, 1982), in which glutamine adds first followed by rapid equilibrium addition of MgATP and then followed by addition of FGAR. This mechanism is consistent with the initial velocity, dead-end inhibition, and substrate/product exchange studies.

The results of these studies differ from the kinetic mechanism for the chicken liver enzyme where glutamine adds first followed by random addition of MgATP and FGAR (Li & Buchanan, 1971) and from the proposed kinetic mechanism for the Erlich ascites tumor cell enzyme in which glutamine adds first followed by release of glutamate, MgATP adds next followed by release of ADP, and FGAR adds last followed by release of FGAM and P_i (Chu & Henderson, 1972).

The ability to reproducibly isolate large amounts of FGAM synthetase, the availability of sequence information, and the preliminary mechanistic studies reported herein provide the framework for future detailed mechanistic and regulatory studies on this intriguing amidotransferase.

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Drosophila Insulin Degrading Enzyme and Rat Skeletal Muscle Insulin Protease Cleave Insulin at Similar Sites†

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ABSTRACT: Insulin degradation is an integral part of the cellular action of insulin. Recent evidence suggests that the enzyme insulin protease is involved in the degradation of insulin in mammalian tissues. *Drosophila*, which has insulin-like hormones and insulin receptor homologues, also expresses an insulin degrading enzyme with properties that are very similar to those of mammalian insulin protease. In the present study, the insulin cleavage products generated by the *Drosophila* insulin degrading enzyme were identified and compared with the products generated by the mammalian insulin protease. Both purified enzymes were incubated with porcine insulin specifically labeled with ^{125}I on either the A19 or B26 position, and the degradation products were analyzed by HPLC before and after sulfitolysis. Isolation and sequencing of the cleavage products indicated that both enzymes cleave the A chain of intact insulin at identical sites between residues A13 and A14 and A14 and A15. Sequencing of the B chain fragments demonstrated that the *Drosophila* enzyme cleaves the B chain of insulin at four sites between residues B10 and B11, B14 and B15, B16 and B17, and B25 and B26. These cleavage sites correspond to four of the seven cleavage sites generated by the mammalian insulin protease. These results demonstrate that all the insulin cleavage sites generated by the *Drosopohila* insulin degrading enzyme are shared in common with the mammalian insulin protease. These data support the hypothesis that there is evolutionary conservation of the insulin degrading enzyme and further suggest that this enzyme plays an important role in cellular function.

Insulin is an important modulator of cellular growth and metabolism. The mechanisms by which insulin exerts its effects on cells are as yet incompletely understood, but certain components of the system have been characterized. The insulin signaling system includes the hormone, a specific receptor on the responding cell membrane, a mechanism for signal transmission that may involve intracellular messengers, and a degradative process for removing and inactivating the hormone.

Both the biological response and degradation of the hormone are initiated by binding of insulin to a specific membrane receptor (Terris & Steiner, 1975). The insulin receptor consists of two subunits, an α subunit which contains the primary binding site and a β subunit which has tyrosine kinase activity and appears to be important in propagating the intracellular signal (Jacobs et al., 1979; Massague et al., 1980; Kasuga et al., 1981; Roth & Cassel, 1983). Subsequent to binding, insulin is internalized via the endosomal pathway and ultimately degraded (Bergeron et al., 1985; Gordon et al., 1980). Some of the membrane-bound insulin is also degraded without requiring rapid internalization (Hamel et al., 1987).

Although the degradative pathway is not as well characterized, many lines of evidence now suggest that a metallo thiol

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