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

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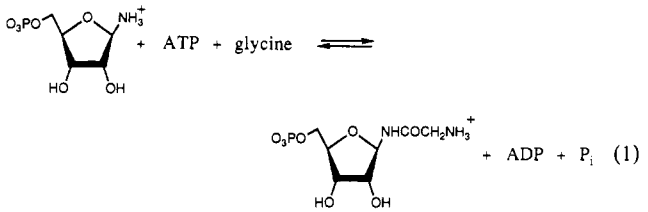
ABSTRACT: The *pur*D gene of *Escherichia coli* encoding the enzyme glycinamide ribonucleotide (GAR) synthetase, which catalyzes the conversion of phosphoribosylamine (PRA), glycine, and MgATP to glycinamide ribonucleotide, MgADP, and P_i, has been cloned and sequenced. The protein, as deduced by the structural gene sequence, contains 430 amino acids and has a calculated M_r of 45 945. Construction of an overproducing strain behind a λpL promoter allowed a 4-fold purification of the protein to homogeneity. N-Terminal sequence analysis and comparison of the sequence with those of other GAR synthetases confirm the amino acid sequence deduced from the gene sequence. Initial velocity studies and product and dead-end inhibition studies are most consistent with a sequential ordered mechanism of substrate binding and product release in which PRA binds first followed by MgATP and then glycine; P_i leaves first, followed by loss of MgADP and finally GAR. Incubation of [^{18}O]glycine, ATP, and PRA results in quantitative transfer of the ^{18}O to P_i. GAR synthetase is very specific for its substrate glycine.

Glycinamide ribonucleotide (GAR) synthetase (EC 6.3.4.13) catalyzes the reversible conversion of phosphoribosylamine (PRA), MgATP, and glycine to GAR, MgADP, and P_i (eq 1). GAR synthetase is of interest for several



reasons. The first is that PRA has a $t_{1/2}$ of 38 s at pH 7.5, 37 °C (Schendel et al., 1988). Recent studies by Srivastava and Bernard (1986) have provided a resurgence of interest in the possibility of channeling of intermediates between consecutive steps in a metabolic pathway. Given the chemical instability of PRA, GAR synthetase, and phosphoribosyl-pyrophosphate (PRPP) amidotransferase, the first two steps in the de novo purine pathway are thought to be prime candidates for the study of protein–protein interactions. To test the possibility of channeling, a detailed kinetic analysis of all the parameters for both of these proteins is required. Purification and characterization of GAR synthetase is an important prerequisite.

The second reason that GAR synthetase is of interest is that nothing is known about the chemical mechanism of this enzyme-catalyzed reaction, although a phosphoanhydride glycine intermediate, in analogy with glutamine synthetase and D-alanine:D-alanine ligase, provides a reasonable working hypothesis (Colanduoni & Villafranca, 1986; Rowe et al., 1969; Duncan & Walsh, 1988). Successful mechanism-based inhibition of these latter two proteins provides promise that similar inhibitors, analogues of glycine, can be easily prepared and will function as potent inhibitors of the purine biosynthetic pathway and hence of DNA biosynthesis. Recent studies using 5,10-dideazatetrahydroaminopterin (Taylor et al., 1985; Inglese et al., 1989) and multisubstrate adducts containing elements of GAR and folate provide examples of potent inhibitors of GAR transformylase with high levels of in vitro cytotoxicity. These studies have rekindled interest in the purine biosynthetic pathway as a target for the design of antitumor agents.

In the past few years the gene sequence of GAR synthetase has been reported from *Drosophila melanogaster* (Henikoff & Furlong, 1986), *Saccaromyces cerevisiae* (Henikoff, 1986), and *Bacillus subtilus* (Ebbole & Zalkin, 1987), but the protein has not been purified to homogeneity from any of these sources. Recent studies from our laboratory in collaboration with the laboratories of Benkovic, Henikoff, and Patterson have succeeded for the first time in purifying to homogeneity GAR synthetase from chicken liver. This activity is part of a novel trifunctional protein, a monomer of M_r = 110K, containing, in addition to GAR synthetase, GAR transformylase and aminoimidazole ribonucleotide (AIR) synthetase activities (Daubner et al., 1985). The *S. cerevisiae* GAR synthetase is postulated to be a bifunctional protein which contains AIR synthetase and GAR synthetase activity, on the basis of gene sequence and partial purification (Fluri et al., 1976). The GAR synthetase from a prokaryotic source, *Pseudomonas aerogenosa*, was partially purified by Nierlich and Magasanik (1965) and shown to be a monomer, M_r 40K. Given that the GAR synthetase from chicken liver is difficult to isolate and only limited amounts are available, efforts were made to clone and sequence the *Escherichia coli* GAR synthetase and to place the gene behind a strong promoter, allowing for overproduction of the protein and for ease of purification of the protein to homogeneity. The successes of these efforts are reported in this paper. In addition, the kinetic mechanism of

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GAR synthetase has been established by utilizing initial velocity and product and dead-end inhibition analyses. Preliminary substrate specificity and mechanistic studies are also reported.

Experimental Procedures

Materials and Methods. Dowex 50W-X8, HEPES, D-ribose, L-glutamine, ninhydrin, ampicillin, phosphoribosyl pyrophosphate, *E. coli* glutaminase (8.4 μmol min^{-1} mg^{-1}), ammonia standard solution, ammonium color reagent, ATP, D-ribose 5-phosphate, bovine serum albumin, L-lactate dehydrogenase (rabbit muscle, 920 μmol min^{-1} mg^{-1}), pyruvate kinase (rabbit muscle, 355 μmol min^{-1} mg^{-1}), hexokinase (yeast, 280 μmol min^{-1} mg^{-1}), glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, 450 μmol min^{-1} mg^{-1}), phosphoenolpyruvate, β-alanine, AMP-PCP, molecular weight standards for gel electrophoresis, ATP–agarose (linked through N-6 of the adenine ring by an eight-carbon spacer, 1.4 μmol of ATP/mL of resin), Sephadex G-25 (fine), Sephadex G-100, and DEAE-Sepharose A-25 were obtained from Sigma Chemical Co. [^{14}C]Glycine (56 mCi/mmol) was obtained from Amersham. [1-^{13}C]Glycine (90.0 atom % ^{13}C) was obtained from MSD Isotopes. $H_2{}^{18}O$ (95.1 atom % ^{18}O) was obtained from Monsanto Research Corp. [1-$^{18}O_2$,^{13}C]-Glycine was prepared by the procedure of Mears and Sobatka (1939). All other reagents used were of reagent grade or better unless otherwise specified. (Aminomethyl)phosphinic acid was prepared by the procedure of Uhing et al. (1961); GAR was prepared by a modification of the procedure of Chettur and Benkovic (1977). 1-Methylaziridinecarboxylate was a gift from Merck Sharp & Dohme Laboratories. 1-Aziridinecarboxylate was prepared by the procedure of Kyburz et al. (1966).

Glycine concentrations were determined by a modified ninhydrin assay with L-glutamine as a standard (Rosen, 1959). The concentration of the glutamine solution was determined by quantitation of the ammonium released by reaction with glutaminase (Williamson & Corky, 1979). Ribose-5-P concentrations were determined by the phosphate (Ames & Dubin, 1960) and orcinol assays (Dische, 1962). Concentration of the protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. All solutions were analyzed for radioactivity in Packard Scint-A scintillation fluid on a Packard 310 liquid scintillation counter. Protein sequencing was done according to the automated Edman degradation procedure by the University of Wisconsin Biotechnology Center. ^{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.

Strains and Media. Strain TX634 [Δ(*lac*), *pur*D215 *rec*A56] was created from strain TX40 (Smith & Gots, 1980) in successive steps: isolation of a stable spontaneous *pur*D mutation, introduction of a srlC300::Tn10 mutation by P1 transduction with subsequent removal, and introduction of the *rec*A56 mutation by cotransduction. Strain TX634 was used for the initial subcloning and complementation analysis of the *pur*H(J)D operon. Strain BSJ72 (obtained from Tom St. John, Fred Hutchinson Cancer Research Center) was used for the *lacZ*$_\alpha$ complementation inactivation identification of subclones and the production of sequencing DNA by superinfection with the M13 helper phage K07 (Vieira & Messing, 1987). Strain TX635 (F′*lacZ*$^+$ *c*I857) contains an episome-borne temperature-sensitive λ repressor and was used as a host for the λpL plasmids (Mieschendahl & Muller-Hill, 1985). Strains were made competent and transformed as described in Alexander at al. (1984). The minimal media of Neidhart et al. (1974) and rich media of Miller (1972) were used for the growth of the *E. coli* K12 strains. The recombinant DNA techniques have been described (Maniatis et al., 1972; Tiedman et al., 1985).

Purification of GAR Synthetase. *E. coli* strain TX635/pJS187 was grown at 30 °C on LB agar plates containing ampicillin. A single colony was used to inoculate 2 L of LB media containing 50 μg/mL ampicillin. The 2 L, at an OD of 2.0, was used to inoculate 24 L of LB media in a New Brunswick microferm fermentor. The bacteria were grown at 30 °C to an OD_{600} = 0.8 (doubling time, 1 h), at which time the media was heat induced to 42 °C and the temperature maintained for 30 min. The temperature was then reduced to 37 °C, and the cells were allowed to grow for an additional 2.5 h. The bacteria were harvested and the cells frozen with liquid nitrogen and stored at −80 °C (yield, 2.5 g/L).

All steps in the purification were performed at 4 °C in 50 mM potassium phosphate buffer (pH 7.5), 5 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride (PMSF). In a typical isolation, 20 g of cells was suspended in 70 mL of buffer. The cells were ruptured in a French pressure apparatus at 16 000 psi, and the cell debris was removed by centrifugation at 12000*g* for 20 min. The supernatant was brought to 0.1% in protamine sulfate by the addition of 1% protamine sulfate solution over a period of 30 min. The mixture was allowed to stir for an additional 30 min and then was centrifuged at 12000*g* for 20 min. The supernatant was brought to 33% saturation in ammonium sulfate (0.196 g/mL) over a 30-min period. Once the addition was complete, the solution was allowed to equilibrate for 30 min and then was centrifuged at 12000*g* for 15 min. The supernatant was brought to 65% saturation in ammonium sulfate (0.214 g/mL) over a period of 30 min. Again the solution was allowed to equilibrate for a period of 30 min and then was centrifuged at 12000*g* for 15 min. The pellet was dissolved in a minimal amount of buffer (~20 mL), centrifuged at 12000*g* for 10 min, and applied to the top of a Sephadex G-25 column (4.5 × 40 cm). The fractions containing protein, $A_{280} > 0.2$ OD, were pooled and immediately applied to a DE-52 column (4.0 × 18 cm) equilibrated in buffer. The column was washed with 250 mL of buffer and then with a 0–300 mM KCl linear gradient (600 × 600 mL) in buffer. The fractions containing GAR synthetase activity were pooled and concentrated in an Amicon ultrafiltration apparatus (PM30 membrane) to a volume of 15 mL. The concentrated protein was centrifuged at 12000*g* for 10 min and then was loaded onto a Sephadex G-100 column (2.5 × 105 cm). The column was eluted with buffer at a flow rate of 13 mL/h. The fractions containing GAR synthetase activity were concentrated by ultrafiltration to a volume of 10 mL. The protein solution was diluted to a volume of 12 mL with buffer and then was stored in 20% glycerol at −80 °C at a concentration of 60–80 mg/mL. Specific activity of GAR synthetase ranged from 19 to 22 μmol min^{-1} mg^{-1} at 37 °C.

Subunit and Native Molecular Weight Determination of GAR Synthetase. Subunit molecular weight was determined from SDS–polyacrylamide gel electrophoresis (10% acrylamide) performed according to the procedure of Laemmli (1970). The molecular weight standards included bovine serum albumin (M_r 66 000), egg albumin (M_r 45 000), erythrocyte carbonic anhydrase (M_r 29 000), trypsinogen (M_r 24 000), and α-lactalbumin (M_r 14 200). The native molecular weight was determined by FPLC sizing chromatography using carbonic anhydrase, egg albumin, bovine serum albumin, and FGAM synthetase (M_r 141 000) as standards.

Enzyme Assays. GAR synthetase was assayed in the forward direction by modifications of the procedure of Schrimsher et al. (1986) using a coupled assay procedure involving pyruvate kinase and lactate dehydrogenase.

All kinetic studies were conducted in the presence of 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM PEP, 0.2 mM NADH, 5 units mL^{-1} lactate dehydrogenase, 4 units mL^{-1} pyruvate kinase, 2 mM magnesium acetate, 0.015–0.03 unit of GAR synthetase, and various concentrations of substrates (PRA, glycine, and MgATP) and inhibitors at 18 °C. Commercially obtained pyruvate kinase was dialyzed against 50 mM Tris-HCl (pH 8.0) and 25% glycerol, and lactate dehydrogenase was dialyzed against 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 25% glycerol. All reaction ingredients except PRA were preincubated at 18 °C for 3 min, and the reaction was initiated by the addition of PRA. (The preparation of PRA is described subsequently). Initial velocities were calculated from the decrease in the absorbance at 340 nm (full scale on the recorder is 0.2 absorbance unit) with $\epsilon = 6200$ M^{-1} cm^{-1}. The reaction temperature was maintained at 18 °C by a Llauda constant-temperature water circulator. Due to the rapid hydrolysis of PRA under the reaction conditions and its low K_m value, kinetic studies using PRA (20–950 μM) were performed in cuvettes (2 mL) with 2.0-cm light paths.

GAR synthetase was assayed in the reverse direction with a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase. A typical assay mixture contained in a total volume of 0.5 mL the following: 100 mM Tris-HCl (pH 8.0), 12 mM $Mg(OAc)_2$, 2.5 mM glucose, 2.4 mM $NADP^+$, 3 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, and 0.03–0.09 unit of GAR synthetase. A stock solution of GAR synthetase was prepared by diluting 10 units of enzyme into 6 mL of 50 mM Tris-HCl (pH 8.0) containing 1 mg mL^{-1} BSA.

Formation of PRA. In order to quantitate the amount of PRA, known amounts of ribose-5-P (71 mM) and NH_4Cl (94 mM) [or ribose-5-P (15 mM) and NH_4Cl (576 mM)] were incubated in a sealed vial at 37 °C, pH 10–11, for 40 min. The amount of PRA was calculated by using the following equation (Schendel et al., 1988):

$$[PRA]_{eq}/[\text{ribose-5-P}]_{eq}[NH_3]_{eq} = 2.6\ M^{-1} \quad (2)$$

Data Analysis. The Michaelis constants for PRA, MgATP, and glycine in the forward direction and β-GAR, MgADP, and P_i in the reverse direction were determined by varying the concentration of one substrate at saturating levels of the other two and fitting the data by nonlinear least-squares regression analysis to eq 3. The initial velocity data were obtained in

$$v = VA/K + A \quad (3)$$

each direction by varying the concentrations of two substrates from $0.2K_m$ to $5K_m$ while holding the third substrate concentration constant at $1.5K_m$ to $2.0K_m$. The initial velocity data were fit to eq 4, or the corresponding equation with appropriate terms missing, to determine the best fit to the steady-state kinetic model (Viola & Cleland, 1982).

$$v = VABC/[\text{constant} + (\text{coeff})A + (\text{coeff})B + (\text{coeff})C + K_aBC + K_bAC + K_cAB + ABC] \quad (4)$$

Inhibition studies were analyzed with the computer programs of Cleland (1979). Data corresponding to competitive, noncompetitive, and uncompetitive were fit to eq 5–7.

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

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

$$v = VA/[K_a + A(1 + I/K_{is})] \quad (7)$$

^{18}O Transfer Experiment. A 0.5-mL solution (30% D_2O) containing 100 mM ATP, 100 mM $MgCl_2$, 200 mM ribose 5-phosphate, 400 mM NH_4Cl, 60 mM [$^{16}O/^{18}O$]glycine (1:1 in isotopic label), and 50 mM Tris-HCl (pH 8.0) was filtered through a glass fiber filter (Whatman GF/A) into a 5-mm NMR tube, and the 80.9-MHz ^{31}P NMR spectrum was recorded. GAR synthetase, 4.6 units, was added, and the NMR tube was incubated at 37 °C for 90 min. When the reaction was complete, as determined by ^{31}P NMR, EDTA was added to give a final concentration of 10 mM. The 80.9-mHz ^{31}P NMR was then recorded with a sweep width of 1000 Hz, quadrature phase detection, a 70° pulse angle, and a 16K data block. A total of 500 acquisitions were obtained with an acquisition time of 3.7 s and a pulse delay of 1 s. To enhance resolution, exponential multiplication with a line-broadening factor of 0.2 Hz was applied to the FID before Fourier transformation.

Results and Discussion

Cloning of the purH(J)D Operon. Initially, λ phages containing the *pur*D gene and flanking DNA were isolated by complementation of a Δ[*pur*H(J)D] λ lysogen strain. The *E. coli* K12 gene bank employed was constructed in the λSE6 vector by Elledge and Walker (1985). After purification, λ DNA was prepared from a representative clone, partially digested with *Sau*3A, and ligated into the *Bam*HI site of plasmid pUC12 (Messing, 1983). Plasmids containing the intact *pur*D gene were identified by complementation. Subsequent characterization of several plasmids by restriction and deletion analysis indicated that a *Sac*I site was present in the *pur*D gene. The largest restriction fragment characterized (2.9 kb, plasmid pJS86) that complemented the *pur*D mutation of strain TX634 was initially sequenced. The restriction fragment was transferred to the phagemid vector Bluescript SK^+ (Stratagene, Inc.) via the *Bam*HI (one site was recreated by one *Sau*3A site) and *Pst*I restriction sites to create plasmid pJS168.

Construction of an Expression Vector for GAR Synthetase. An expression vector for GAR synthetase was created by subcloning a restriction fragment containing the *pur*D structural gene into a plasmid containing the λpL promoter. The restriction sites employed were an *Ase*I site at nucleotide 1961 and a *Pst*I site in the flanking polylinker. After *Ase*I digestion of plasmid pJS168, the resulting restriction fragments were treated with T4 DNA polymerase to create blunt ends (Maniatis et al., 1982). Following the *Pst*I digestion, a 1.6-kb fragment containing the *Pur*D gene was recovered from a 0.7% agarose gel and ligated into the *Sma*I–*Pst*I sites of plasmid pJS88. Following transformation into strain TX635, the desired plasmids were identified by restriction digest. A representative plasmid, designated pJS187, was retained for expression studies. Plasmid pJS88 is a λpL expression vector similar to the ones described by Remaut et al. (1981). Strain TX635 was also used as the host, and synthesis of GAR synthetase was induced by a temperature shift to 42 °C.

DNA Sequence Analysis. The sequence of the 2.9-kb DNA fragment containing the *pur*D gene was determined by the dideoxy chain termination method (Sanger et al., 1979). Overlapping deletion subclones from both strands for DNA sequencing (Figure 1) were generated by the procedure of Henikoff (1987). Analysis of the completed sequence revealed that the initial portion of the *pur*H(J) gene and the associated *pur*H(J)D control region was lacking. Accordingly, a new subcloning by the miniMu procedure (Groisman et al., 1984)

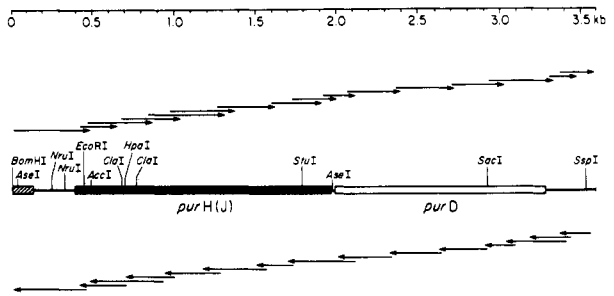

FIGURE 1: Sequencing strategy for *pur*D. The location of the 6-bp restriction enzyme sites used in subcloning and sequencing studies of the *pur*H(J)D operon are indicated. The arrows denote sequencing direction, and the length of the arrow is proportional to the number of nucleotides determined. The *pur*D coding region extends from nucleotide 1977 to nucleotide 3263 and is indicated by the open box. The characterization of the *pur*H(J) gene and control region will be reported elsewhere (Flannigan et al., unpublished results).

was undertaken to recover the missing promoter proximal portion of the *pur*H(J)D operon. The final DNA sequence for the *pur*H(J)D operon was derived from a 3.55-kb DNA fragment. The *pur*D portion is reported here, and the *pur*H(J) portion will be reported elsewhere (Flannigan et al., unpublished results). The DNA sequences were compiled and analyzed by the facilities of the BIONET resource.

Derived Amino Sequence. After the DNA sequence of the 3.55-kb DNA fragment containing the *pur*H(J)D operon was determined, it was analyzed for open reading frames. Two were found potentially encoding polypeptides of M_r 57 335 and 45 945. The M_r 57 335 polypeptide was identified as the bifunctional aminoimidazolecarboxamide ribonucleotide transformylase and IMP cyclohydrolase (Flannigan et al., unpublished results). The second open reading frame (*pur*D) was identified as encoding GAR synthetase by the following criteria: (1) complementation of *E. coli pur*D mutants, (2) the presence of a *Sac*I restriction site in the coding region, and (3) comparison to the amino-terminal sequence obtained by automated Edman degradation of purified GAR synthetase (eq 8). An additional confirmation of the correctness of the *E. coli* GAR synthetase sequence is indicated by comparison to the GAR synthetase sequences from other organisms (see below).

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Edman degradation:   M-K-V-L-V-I-G-N-G-G-R-E-H-A-L
                                                    (8)
gene:                M-K-V-L-V-I-G-N-G-G-R-E-H-A-L
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The *pur*D coding region extends from nucleotide 1977 to nucleotide 3263 (Figure 2) and is preceded by a good ribosomal initiation site (Shine & Dalgarno, 1974), GGAG, at ncleotides 1967–1970 (Figure 2). The amino-terminal sequence of the purified enzyme shows that the initiator methionine is not processed (eq 8).

Forty-seven base pairs downstream of the *pur*D coding region is a region of dyad symmetry (nucleotides 3309–3351) with the characteristics of a ρ-independent terminator that could function in the termination of *pur*H(J)D transcription. It has a ΔG of −26.1 kcal/mol as calculated by the program of Zuker and co-workers (Jacobson et al., 1984).

Comparison to Other GAR Synthetases. The genes encoding GAR synthetase have sequenced from *B. subtilis* (Ebbole & Zalkin, 1987), *S. cerevisiae* (Henikoff, 1986), *D. melanogaster* (Henikoff & Furling, 1983), and *Drosophila pseudoobscura* (Henikoff & Eghtedarzadeh, 1987). As in *E. coli*, GAR synthetase from *B. subtilis* contains a single activity. However, in *S. cerevisiae* GAR synthetase is part of a bifunctional polypeptide with AIR synthetase, and in the two *Drosophila* species investigated, GAR synthetase is part of a trifunctional polypeptide that includes AIR synthetase and GAR transformylase. In both *Drosophila* and *S. cerevisiae*, GAR synthetase is the amino-terminal activity on the corresponding multifunctional polypeptide. The alignment of the deduced *E. coli* GAR synthetase sequence with the GAR synthetase sequences from these other organisms is shown in Figure 3. Overall, GAR synthetase from *E. coli* shows the greatest similarity to the GAR synthetase of *B. subtilis* and the least with the *S. cerevisiae* enzyme.

Table I: Isolation of GAR Synthetase

	sp act. (μmol min^{-1} mg^{-1}), 25 °C	total units (μmol/min)	total protein (mg)
crude[a]	4.95	13 700	2764
protamine sulfate	5.12	14 200	2777
33% $(NH_4)_2SO_4$	5.31	13 300	2502
G-25	8.94	13 200	1482
DE-52	12.8	12 472	971
G-100	15.0	13 500	900
final	22[b]		

[a] 20.1 g of cells. [b] At 37 °C.

Isolation and Characterization of E. coli GAR Synthetase. GAR synthetase from *E. coli* strain TX635 containing plasmid pJS187 has been purified 4-fold to homogeneity, Table I. Twenty grams of bacteria typically yield 900 mg of protein. SDS gel electrophoresis (Figure 4) and FPLC sizing chromatography both indicate by comparison with standards of known molecular weight that GAR synthetase is a monomer of M_r 46 800, closely corresponding to M_r 45 945 as determined by the gene sequence.

Transfer of ^{18}O from Labeled Glycine to P_i. The conversion of glycine to GAR presumably involves acylation of the carboxylate of glycine to form the mixed anhydride $^{-}O_3POCOCH_2NH_3^+$. To test this hypothesis, a 1:1 mixture of [1-$^{16}O_2$,^{13}C]glycine and [1-$^{18}O_2$,^{13}C]glycine was prepared (Figure 5a) and incubated with GAR synthetase, MgATP, ribose-5-P, and ammonium chloride. The reaction was monitored by ^{31}P NMR spectroscopy, and upon completion of the reaction, the P_i was isolated and its ^{31}P NMR spectrum recorded (Figure 5B). This spectrum indicates that a mixture of [$^{16}O/^{18}O$]P_i is produced with a ratio identical with that of the starting glycine. The ^{18}O from the carboxylate of glycine is thus transferred quantitatively to P_i as predicted.

Kinetic Studies. PRA has been recently shown to have a half-life of 38 s at pH 7.5 and 37 °C. The kinetics of the GAR synthetase reaction in both the forward and the reverse directions were therefore studied at pH 8.0 and 18 °C to minimize the decomposition of PRA, and hence to avoid extensive corrections due to this decomposition. At fixed concentrations of MgATP and MgADP (1.15 mM) no significant inhibition or activation of the initial velocities in the forward or reverse direction of the GAR synthetase catalyzed reaction were observed when the free Mg^{2+} concentration was varied from 0.02 to 5.4 mM in the forward direction or from 0.01 to 12 mM in the reverse direction. Determination of the Michaelis constants for the three substrates in the forward direction resulted in a K_m of 70 ± 13 μM for PRA, 170 ± 40 μM for MgATP, and 270 ± 70 μM for glycine and in the reverse direction in a K_m of 6.4 ± 0.2 μM for MgADP, 30 ± 3 μM for GAR, and 0.54 ± 0.06 mM for P_i.

Initial velocity and product and dead-end inhibition studies were undertaken to determine the order of addition of substrates in each direction. The results from the initial velocity data for both the forward and reverse direction are summarized in Table II. In the forward direction all patterns are inter-

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                                                                  1965       1975
                                                                 CAT TAA TGGAGCAATA
                                                                 His       S/D

          1985        1994        2003        2012        2021
 G ATG AAA GTA TTA GTG ATT GGT AAC GGC GGG CGC GAG CAC GCG CTG GCC TGG
   MET Lys Val Leu Val Ile Gly Asn Gly Gly Arg Glu His Ala Leu Ala Trp

2030        2039        2048        2057        2066        2075
 AAA GCG GCC CAG TCG CCG CTG GTT GAG ACT GTT TTT GTT GCT CCG GGT AAT GCA
 Lys Ala Ala Gln Ser Pro Leu Val Glu Thr Val Phe Val Ala Pro Gly Asn Ala

2084        2093        2102        2111        2120        2129
 GGC ACT GCA CTG GAA CCC GCG CTG CAA AAC GTT GCT ATT GGC GTG ACC GAT ATC
 Gly Thr Ala Leu Glu Pro Ala Leu Gln Asn Val Ala Ile Gly Val Thr Asp Ile

2138        2147        2156        2165        2174        2183
 CCG GCG CTG TTG GAT TTC GCA CAA AAC GAA AAG ATT GAT CTG ACC ATC GTC GGC
 Pro Ala Leu Leu Asp Phe Ala Gln Asn Glu Lys Ile Asp Leu Thr Ile Val Gly

2192        2201        2210        2219        2228        2237
 CCG GAA GCG CCG CTG GTG AAA GGC GTG GTC GAT ACC TTC CGC GCC GCC GGG CTG
 Pro Glu Ala Pro Leu Val Lys Gly Val Val Asp Thr Phe Arg Ala Ala Gly Leu

2246        2255        2264        2273        2282        2291
 AAA ATC TTC GGC CCA ACC GCA GGT GCG GCC CAA CTG GAA GGC TCA AAA GCG TTT
 Lys Ile Phe Gly Pro Thr Ala Gly Ala Ala Gln Leu Glu Gly Ser Lys Ala Phe

2300        2309        2318        2327        2336        2345
 ACC AAA GAT TTC CTG GCC CGC CAT AAG ATC CCT ACG GCG GAA TAC CAG AAC TTC
 Thr Lys Asp Phe Leu Ala Arg His Lys Ile Pro Thr Ala Glu Tyr Gln Asn Phe

2354        2363        2372        2381        2390        2399
 ACC GAG GTA GAA CCT GCG CTG GCG TAT CTG CGT GAG AAA GGC GCG CCA ATC GTC
 Thr Glu Val Glu Pro Ala Leu Ala Tyr Leu Arg Glu Lys Gly Ala Pro Ile Val

2408        2417        2426        2435        2444        2453
 ATT AAA GCG GAC GGT CTG GCT GCC GGG AAA GGC GTT ATC GTG GCG ATG ACG CTG
 Ile Lys Ala Asp Gly Leu Ala Ala Gly Lys Gly Val Ile Val Ala MET Thr Leu

2462        2471        2480        2489        2498        2507
 GAA GAA GCG GAA GCG GCT GTT CAC GAT ATG CTG GCG GGC AAC GCT TTT GGC GAC
 Glu Glu Ala Glu Ala Ala Val His Asp MET Leu Ala Gly Asn Ala Phe Gly Asp

2516        2525        2534        2543        2552        2561
 GCG GGT CAT CGC ATC GTT ATC GAA GAG TTC CTC GAT GGC GAA GAA GCG AGC TTT
 Ala Gly His Arg Ile Val Ile Glu Glu Phe Leu Asp Gly Glu Glu Ala Ser Phe

2570        2579        2588        2597        2606        2615
 ATC GTG ATG GTG GAC GGC GAG CAT GTG CTG CCG ATG GCT ACC AGC CAG GAT CAC
 Ile Val MET Val Asp Gly Glu His Val Leu Pro MET Ala Thr Ser Gln Asp His

2624        2633        2642        2651        2660        2669
 AAA CGC GTA GGC GAT AAA GAT ACC GGA CCA AAC ACC GGC GGG ATG GGC GCT TAC
 Lys Arg Val Gly Asp Lys Asp Thr Gly Pro Asn Thr Gly Gly MET Gly Ala Tyr

2678        2687        2696        2705        2714        2723
 TCC CCC GCG CCG GTA GTA ACC GAT GAC GTT CAT CAG CGC ACC ATG GAA CGT ATT
 Ser Pro Ala Pro Val Val Thr Asp Asp Val His Gln Arg Thr MET Glu Arg Ile

2732        2741        2750        2759        2768        2777
 ATC TGG CCA ACC GTG AAA GGC ATG GCG GCG GAA GGC AAC ACC TAC ACC GGT TTT
 Ile Trp Pro Thr Val Lys Gly MET Ala Ala Glu Gly Asn Thr Tyr Thr Gly Phe

2786        2795        2804        2813        2822        2831
 CTC TAC GCG GGC CTG ATG ATC GAC AAA CAG GGC AAT CCG AAG GTT ATT GAA TTT
 Leu Tyr Ala Gly Leu MET Ile Asp Lys Gln Gly Asn Pro Lys Val Ile Glu Phe

2840        2849        2858        2867        2876        2885
 AAC TGC CGC TTT GGC GAT CCG GAA ACC CAG CCG ATT ATG CTG CGC ATG AAG TCC
 Asn Cys Arg Phe Gly Asp Pro Glu Thr Gln Pro Ile MET Leu Arg MET Lys Ser

2894        2903        2912        2921        2930        2939
 GAT CTG GTT GAG CTC TGC CTG GCG GCC TGT GAA AGC AAA CTG GAC GAG AAA ACG
 Asp Leu Val Glu Leu Cys Leu Ala Ala Cys Glu Ser Lys Leu Asp Glu Lys Thr
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2948        2957        2966        2975        2984        2993
 TCC GAG TGG GAT GAA CGC GCT TCT CTC GGC GTG GTG ATG GCT GCG GGT GGA TAT
 Ser Glu Trp Asp Glu Arg Ala Ser Leu Gly Val Val MET Ala Ala Gly Gly Tyr

3002        3011        3020        3029        3038        3047
 CCG GGT GAT TAC CGC ACC GGT GAC GTG ATC CAC GGC CTG CCG CTG GAA GAA GTG
 Pro Gly Asp Tyr Arg Thr Gly Asp Val Ile His Gly Leu Pro Leu Glu Glu Val

3056        3065        3074        3083        3092        3101
 GCA GGC GGC AAA GTG TTC CAC GCG GGC ACA AAA CTG GCG GAT GAC GAG CAG GTA
 Ala Gly Gly Lys Val Phe His Ala Gly Thr Lys Leu Ala Asp Asp Glu Gln Val

3110        3119        3128        3137        3146        3155
 GTG ACC AAC GGC GGG CGC GTA CTG TGC GTC ACC GCG CTG GGT CAT ACC GTG GCA
 Val Thr Asn Gly Gly Arg Val Leu Cys Val Thr Ala Leu Gly His Thr Val Ala

3164        3173        3182        3191        3200        3209
 GAA GCG CAG AAA CGC GCC TAT GCC TTA ATG ACC GAT ATC CAC TGG GAC GAC TGC
 Glu Ala Gln Lys Arg Ala Tyr Ala Leu MET Thr Asp Ile His Trp Asp Asp Cys

3218        3227        3236        3245        3254        3263            3276
 TTC TGC CGG AAA GAT ATC GGC TGG CGC GCT ATC GAA CGC GAG CAG AAC TAA CGCGACAGTT
 Phe Cys Arg Lys Asp Ile Gly Trp Arg Ala Ile Glu Arg Glu Gln Asn

      3286        3296        3306        3316        3326        3336        3346
 TTGCCAATAG CGTTTTGCGC GTGATCCCTA ACTGACGGGC GGCTTCGGTT TTGTTGCCGC CCGTTTTCTC

      3356        3366        3376        3386        3396        3406        3416
 CAGCGCCGCC AGAATCACCT CTTTTTCCAC TTCCACCAAC GGCTGAATAT CCTGACTTTG TCCCAGCGGG

      3426        3436        3446        3456        3466        3476        3486
 ATCGGCGTAC TGGCAATCGC CAGCGGCAGC TCGCGTTCGG AAATATATTC CCCGGTCAGC AGCACCACTG

      3496        3506        3516        3526        3536        3546
 CCCGTTCCAC CGCGTTTTCC AGCTCACGAA TATTTCCCGG CCAGTCGTAA TGAATCAACA GATC
```

FIGURE 2: Nucleotide and deduced amino acid sequences of *pur*D. The DNA sequence of the sense strand of the *pur*D gene is shown. The numbering scheme continues from the upstream sequence of the *pur*H(J) gene and is shown from an *Ase*I site at nucleotide 1961 to a *Sau*3A site at nucleotide 3550. The proposed *pur*D Shine–Dalgarno (S/D) sequence at nucleotides 1967–1970 is underlined. The *Ase*I restriction site contains the termination codon of the upstream *pur*H(J) coding region. The amino-terminal GAR synthetase residues confirmed by sequencing are overlined. 47 bp downstream from the *pur*D coding region is a region of dyad symmetry (nucleotides 3309–3351) which is overlined. This region of dyad symmetry has a ΔG of −26.1 kcal/mol as calculated with the program of Jacobsen et al. (1984) and could represent the termination site of *pur*H(J)D transcription.

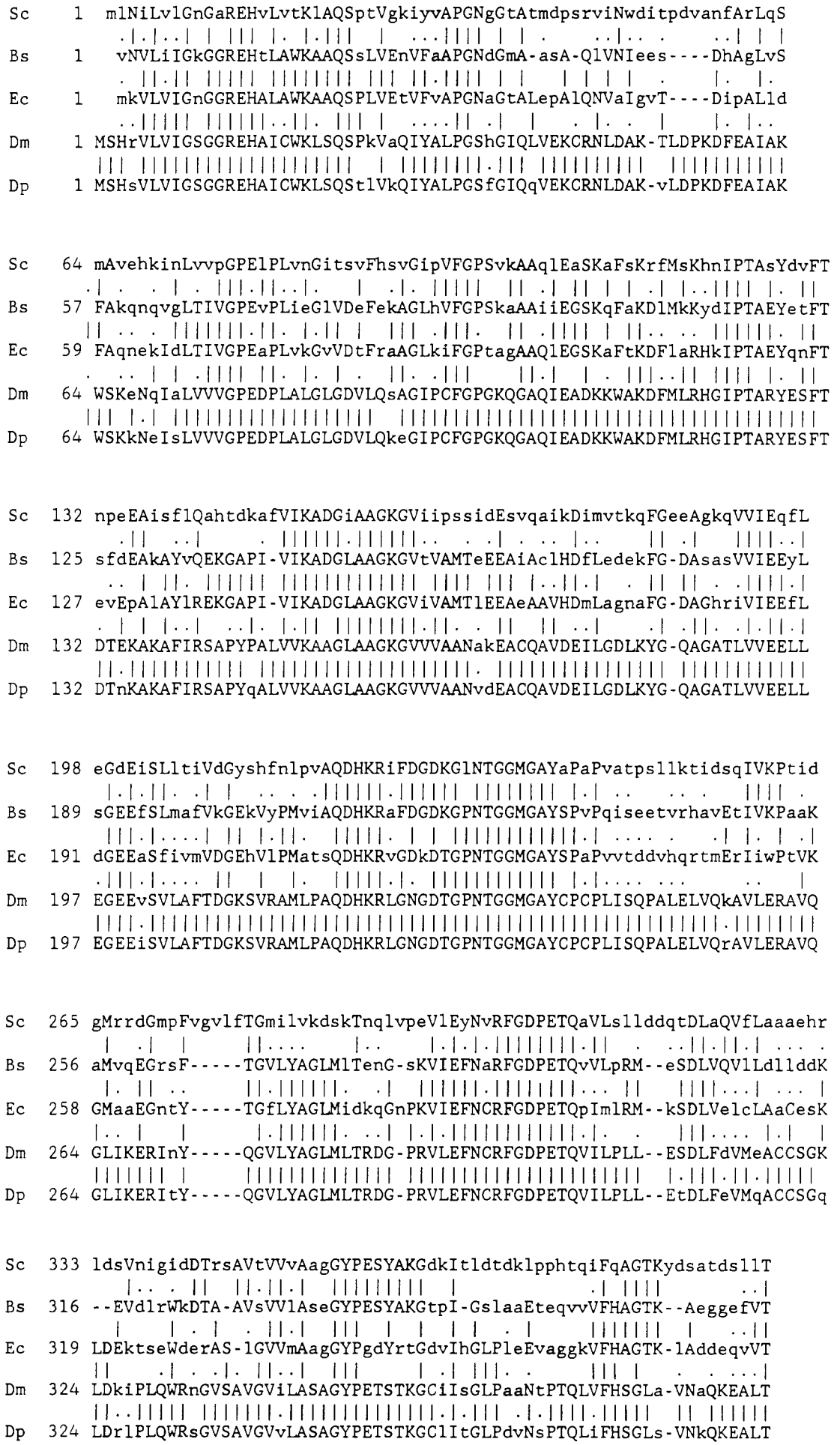
Table II: Initial Velocity Patterns

Forward Direction					
varied substrates	fixed substrates	pattern type	apparent Michaelis constants (μM) MgATP	Gly	PRA
MgATP vs Gly	PRA (151 μM)	intersecting	171 ± 12.0	78.6 ± 10	
MgATP vs PRA	Gly (1 mM)	intersecting	177 ± 10.1		79.0 ± 4.5
PRA vs Gly	MgATP (392 μM)	intersecting		350 ± 27	35.2 ± 2.8
PRA vs Gly	MgATP (1.96 mM)	parallel		185 ± 14	32.0 ± 2.7
Reverse Direction					
varied substrates	fixed substrates	pattern type	apparent Michaelis constants (μM) ADP	GAR	P_i
MgADP vs GAR	P_i (980 μM)	intersecting	5.2 ± 0.9	39.8 ± 5.5	
MgADP vs P_i	GAR (66 μM)	intersecting	3.0 ± 0.9		669 ± 117
GAR vs P_i	MgADP (12.8 μM)	intersecting		36.6 ± 8.4	1188 ± 224
GAR vs P_i	MgADP (128 μM)	parallel		27.4 ± 6.7	526 ± 135

secting when the third substrate is held constant at a concentration of $2K_m$. However, the PRA vs glycine pattern changes from an intersecting to a parallel pattern when the concentration of the third substrate is changed from 184 μM (subsaturating) to 1.96 mM (saturating). Intersecting patterns are obtained with PRA vs MgATP at saturating levels of glycine ($4K_m$ of glycine, 1 mM) and with MgATP vs glycine at saturating levels of PRA ($4K_m$, 315 μM). These results indicate that the kinetic mechanism is sequential, that is, no products are released before all of the substrates have added to the enzyme, and that MgATP binds between PRA and glycine.

In the reverse direction all patterns are again intersecting when the third substrate is held constant at $2K_m$. The GAR vs P_i pattern changes from an intersecting to a parallel pattern when the concentration of the third substrate MgADP is changed from 12.8 μM (subsaturating) to 0.13 mM (saturating). Intersecting lines are obtained with GAR vs MgADP at saturating levels of P_i (11.8 mM) and with MgADP vs P_i at saturating levels of GAR (0.60 mM). These results again indicate the reverse reaction is also sequential and that MgADP binds between GAR and P_i.

Dead-End Inhibition Patterns. In an attempt to determine if the sequential mechanism is ordered or random in the


Sc 1 mlNiLvlGnGaREHvLvtKlAQSptVgkiyvAPGNgGtAtmdpsrviNwditpdvanfArLqS
Bs 1 vNVLiIGkGGREHtLAWKAAQSsLVEnVFaAPGNdGmA-asA-QlVNIees----DhAgLvS
Ec 1 mkVLVIGnGGREHALAWKAAQSPLVEtVFvAPGNaGtALepAlQNVaIgvT----DipALld
Dm 1 MSHrVLVIGSGGREHAICWKLSQSPkVaQIYALPGShGIQLVEKCRNLDAK-TLDPKDFEAIAK
Dp 1 MSHsVLVIGSGGREHAICWKLSQStlVkQIYALPGSfGIQqVEKCRNLDAK-vLDPKDFEAIAK
Sc 64 mAvehkinLvvpGPElPLvnGitsvFhsvGipVFGPSvkAAqlEaSKaFsKrfMsKhnIPTAsYdvFT
Bs 57 FAkqnqvgLTIVGPEvPLieGlVDeFekAGLhVFGPSkaAAiiEGSKqFaKDlMkKydIPTAEYetFT
Ec 59 FAqnekIdLTIVGPEaPLvkGvVDtFraAGLkiFGPtagAAQlEGSKaFtKDFlaRHkIPTAEYqnFT
Dm 64 WSKeNqIaLVVVGPEDPLALGLGDVLQsAGIPCFGPGKQGAQIEADKKWAKDFMLRHGIPTARYESFT
Dp 64 WSKkNeIsLVVVGPEDPLALGLGDVLQkeGIPCFGPGKQGAQIEADKKWAKDFMLRHGIPTARYESFT
Sc 132 npeEAisflQahtdkafVIKADGiAAGKGViipssidEsvqaikDimvtkqFGeeAgkqVVIEqfL
Bs 125 sfdEAkAYvQEKGAPI-VIKADGLAAGKGVtVAMTeEEAiAclHDfLedekFG-DAsasVVIEEyL
Ec 127 evEpAlAYlREKGAPI-VIKADGLAAGKGViVAMTlEEAeAAVHDmLagnaFG-DAGhriVIEEfL
Dm 132 DTEKAKAFIRSAPYPALVVKAAGLAAGKGVVVAANakEACQAVDEILGDLKYG-QAGATLVVEELL
Dp 132 DTnKAKAFIRSAPYqALVVKAAGLAAGKGVVVAANvdEACQAVDEILGDLKYG-QAGATLVVEELL
Sc 198 eGdEiSLltiVdGyshfnlpvAQDHKRiFDGDKGlNTGGMGAYaPaPvatpsllktidsqIVKPtid
Bs 189 sGEEfSLmafVkGEkVyPMviAQDHKRaFDGDKGPNTGGMGAYSPvPqiseetvrhavEtIVKPaaK
Ec 191 dGEEaSfivmVDGEhVlPMatsQDHKRvGDkDTGPNTGGMGAYSPaPvvtddvhqrtmErIiwPtVK
Dm 197 EGEEvSVLAFTDGKSVRAMLPAQDHKRLGNGDTGPNTGGMGAYCPCPLISQPALELVQkAVLERAVQ
Dp 197 EGEEiSVLAFTDGKSVRAMLPAQDHKRLGNGDTGPNTGGMGAYCPCPLISQPALELVQrAVLERAVQ
Sc 265 gMrrdGmpFvgvlfTGmilvkdskTnqlvpeVlEyNvRFGDPETQaVLsllddqtDLaQVfLaaaehr
Bs 256 aMvqEGrsF-----TGVLYAGLMlTenG-sKVIEFNaRFGDPETQvVLpRM--eSDLVQVlLdlldK
Ec 258 GMaaEGntY-----TGfLYAGLMidkqGnPKVIEFNCRFGDPETQpImlRM--kSDLVelcLAaCesK
Dm 264 GLIKERInY-----QGVLYAGLMLTRDG-PRVLEFNCRFGDPETQVILPLL--ESDLFdVMeACCSGK
Dp 264 GLIKERItY-----QGVLYAGLMLTRDG-PRVLEFNCRFGDPETQVILPLL--EtDLFeVMqACCSGq
Sc 333 ldsVnigidDTrsAVtVVvAagGYPESYAKGdkItldtdklpphtqiFqAGTKydsatdsllT
Bs 316 --EVdlrWkDTA-AVsVVlAseGYPESYAKGtpI-GslaaEteqvvVFHAGTK--AeggefVT
Ec 319 LDEktseWderAS-lGVVmAagGYPgdYrtGdvIhGLPleEvaggkVFHAGTK-lAddeqvVT
Dm 324 LDkiPLQWRnGVSAVGViLASAGYPETSTKGCiIsGLPaaNtPTQLVFHSGLa-VNaQKEALT
Dp 324 LDrlPLQWRsGVSAVGVvLASAGYPETSTKGClItGLPdvNsPTQLiFHSGLs-VNkQKEALT

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Sc  396 NGGRVlsVTstaqdlrtAvDtVYeAVkcvhfqnsy--yRKDIayRAf
        |||||. ||   . .  | | || ||  ..    .  .|||| .||.
Bs  373 NGGRVanVTAfdeTfeaArDRVYkAVdeIfkpglF--fRKDIGaRAlkAaQk
        |||||. |||.  |.  |  |.| .. .|.   .|  .|||||.||.   |
Ec  380 NGGRVLcVTALghTvaEAqkRAYaLmtdIhwddcF--cRKDIGwRAiereQn
        ||||||.. ||  .. ||  .|  |.  | .      .| ||  .|
Dm  386 NGGRVLIAIALDgSLKEAAAKATKLAGsIsFsGsGAQYRTDIAQKAFKIAsAstP
        |||||||||||| ||||||||||||.|.| |.|||||||||||||| |. |
Dp  386 NGGRVLIAIALDaSLKEAAAKATKLAGtItFaGtGAQYRTDIAQKAFKIAiAtaP
```

FIGURE 3: Homology with other GAR synthetases. The *E. coli* (Ec) sequence was taken from Figure 2, and the GAR synthetase sequences of *B. subtilis* (Bs) (Ebbole & Zalkin, 1987), *S. cerevisiae* (Sc) (Henikoff, 1986), *D. melanogaster* (Dm) (Henikoff & Furlong, 1983), and *D. pseudoobscura* (Dp) (Henikoff & Eghtedarzadeh, 1987) have been reported. The sequence were aligned by the GENALIGN program of Martinez (1988) as implemented on BIONET, and local adjustments were made by visual inspection. All sequences start with residue 1, and the numbering scheme does not include gaps. The (|) symbols indicate identity while the (·) symbols indicate conserved residues according to the system of Amuro et al. (1985).

Table III. Dead-End Inhibition of *E. coli* GAR Synthetase

variable substrate	fixed substrates	inhibitor	inhibition pattern	K_{is}	K_{ii}
ATP	PRA (302 μM), Gly (300 μM)	AMP-PCP	C	135 ± 12 μM	
Gly	PRA (92 μM), ATP (166 μM)	AMP-PCP	NC	279 ± 135 μM	486 ± 156 μM
PRA	ATP (166 μM), Gly (192 μM)	AMP-PCP	UC		161 ± 16 μM
ATP	Gly (132 μM), PRA (93 μM)	β-alanine	UC		43.2 ± 2.6 mM
Gly	PRA (93 μM), ATP (166 μM)	β-alanine	C	36.0 ± 5.0 mM	
PRA	ATP (166 μM), Gly (200 μM)	β-alanine	UC		78.9 ± 3.7 mM

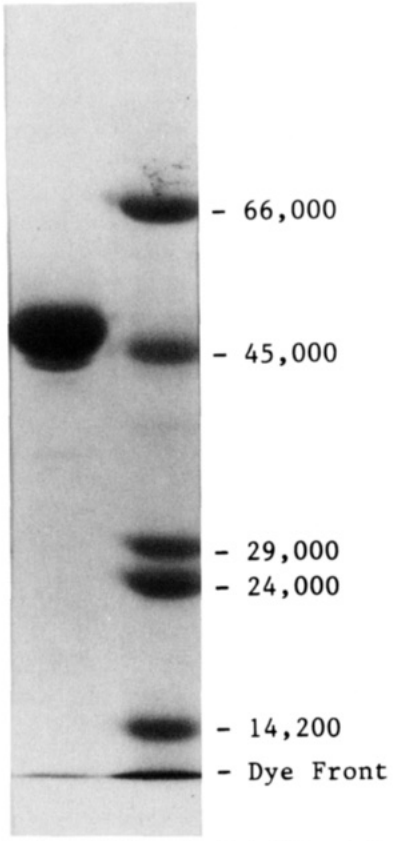
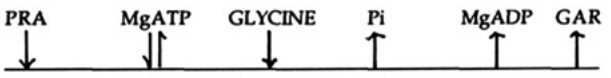


FIGURE 4: SDS gel (10% acrylamide) of *E. coli* GAR synthetase: lane 1, 35 μg of GAR synthetase; lane 2, molecular weight standards.

forward direction and, if the former, which substrate binds first, dead-end inhibition studies were undertaken. The inhibition patterns with the ATP analogue AMP-PCP and the glycine analogue β-alanine for the forward reaction are shown in Table III. All patterns give linear inhibition. The apparent slope and intercept inhibition constants are from fits of the data to eq 5–7. In this study, both AMP-PCP and β-alanine are uncompetitive vs PRA. AMP-PCP is noncompetitive vs glycine, and β-alanine is uncompetitive vs MgATP. These patterns observed with AMP-PCP provide evidence that

Scheme I



MgATP binds before glycine but after PRA. The β-alanine, dead-end inhibitor used to mimic glycine is competitive vs glycine and uncompetitive vs MgATP and PRA. However, the inhibition constants of β-alanine are very large (ranging from 36 to 79 mM), and therefore, the data are not as accurate as the AMP-PCP studies. In a control experiment, the same patterns were repeated with KCl (0–80 mm) replacing β-alanine. No significant effect on the initial velocities were observed, suggesting that the observed effects with β-alanine are not due to altered ionic strength. These studies also support the conclusion that glycine binds subsequent to MgATP and PRA.

A final control experiment was performed to ensure that the concentrations of ribose-5-P and ammonium salts required to generate PRA did not inhibit the GAR synthetase reaction in the forward direction. The concentration of NH_4Cl was varied from 0.15 to 6.65 mM, and no significant effect on the rates was observed. Ribose-5-P was shown to be a linear competitive inhibitor of PRA with K_{is} = 1.22 ± 0.21 mM. Thus, neither of these compounds present in the PRA-generating solution interfered with the kinetic determinations described above.

Product Inhibition Studies. In an attempt to determine if the sequential mechanism is random or ordered in the reverse direction and, if ordered, which substrate binds first, product inhibition studies were undertaken. PRA is a potent competitive inhibitor of GAR with a K_{is} = 3.1 ± 0.2 μM. Since PRA binds first in the forward direction, these results imply that GAR binds first in the reverse direction. Unfortunately at this time no good dead-end inhibitors of GAR, P_i, or MgADP have been found which do not interfere with the coupled assay, precluding further analysis using this method.

Kinetic Mechanism. The kinetic mechanism that is most consistent with the above data from both forward and reverse initial velocity studies and the product and dead-end inhibition studies is the sequential, ordered mechanism shown in Scheme I. To investigate this proposed ordered reaction mechanism

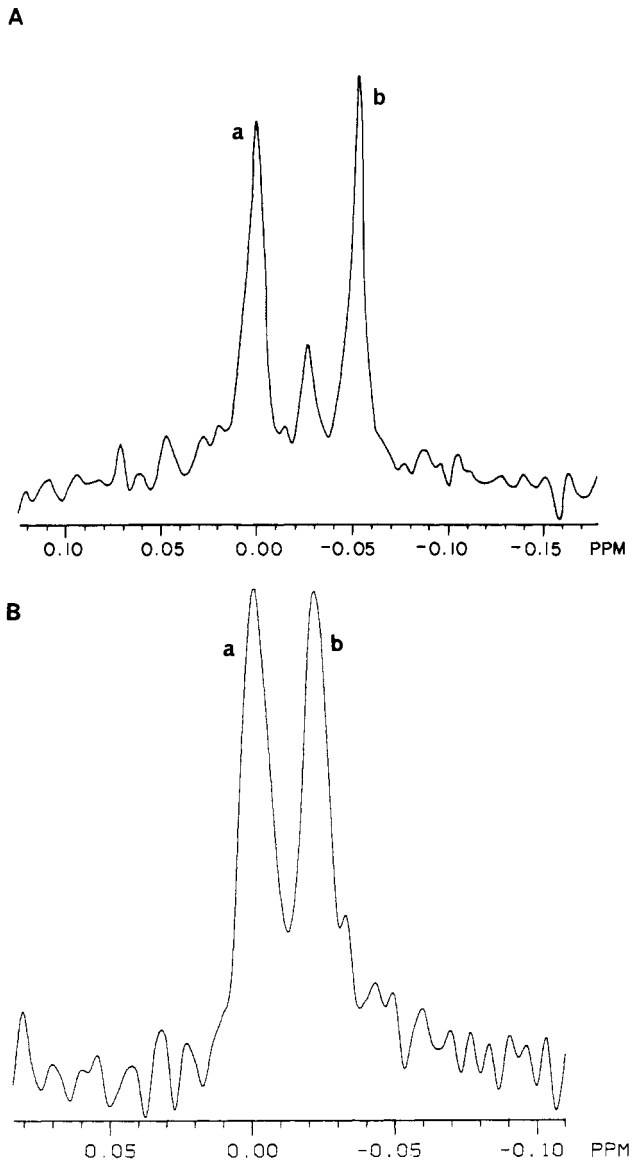


FIGURE 5: (A) A 50.3-MHz ^{13}C NMR spectrum of a mixture of [1-^{13}C,$^{18}O_2$]glycine (peak b) and [1-^{13}C,$^{16}O_2$]glycine (peak a). The peak in the center is [1-^{13}C,^{16}O,^{18}O]glycine. The spectrum was obtained from 100 acquisitions with broad-band proton decoupling with a 35° pulse angle, 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 inorganic phosphate produced from the reaction of GAR synthetase, MgATP, PRA, and a 1:1 mixture of [^{18}O]glycine and [^{16}O]glycine. Peak a is [^{16}O]P_i, and peak b is [^{18}O]P_i.

in the forward direction in greater detail, data obtained from varying two substrates (glycine and MgATP) at two different levels of PRA (151 and 315 μM) were fit to Cleland's initial velocity programs for ter reactant systems (Viola & Cleland, 1982). The mechanism giving the best fit in the forward direction is indicated in Scheme I and describes an equilibrium ordered addition of PRA and MgATP which occurs to form an E·PRA·MgATP binary complex followed by addition of glycine.

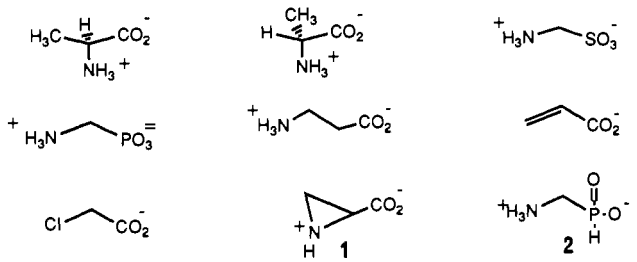
The kinetic data in sum favor the mechanism outlined in Scheme I in which, subsequent to the ordered addition of PRA, MgATP, and glycine, P_i is released followed by MgADP and finally GAR.

Substrate Specificity. The mechanisms of glutamine synthetase and more recently D-alanine:D-alanine ligase involve activation of the carboxylate of the amino acid by phosphorylation with ATP to form a mixed anhydride followed by reaction with an amine to form an amide (eq 9). GAR

$$RCO_2^- + ATP \rightleftarrows RCO_2PO_3 + ADP$$
$$RCO_2PO_3 + NH_2R' \rightleftarrows RCONH_2R' + P_i \quad (9)$$

synthetase presumably proceeds via a similar mechanism. Extensive substrate specificity studies on glutamine synthetase and D-alanine:D-alanine ligase suggest that one ought to be able to design analogues of the amino acid which would function as potent inhibitors (Duncan & Walsh, 1988; Morrison & Walsh, 1987). The glycine substrate specificity of GAR synthetase has been investigated in some detail (Chart I).

Chart I: Glycine Analogues Investigated as Inhibitors or Substrates of GAR Synthetase



None of the compounds are potent competitive inhibitors with respect to glycine or irreversible inhibitors with respect to turnover. Furthermore, use of the coupled assay with pyruvate kinase and lactate dehydrogenase and 100 times the normal concentration of GAR synthetase failed to show any ADP production with either 2-aziridinecarboxylate (**1**) or (aminomethyl)phosphinic acid (**2**), indicating that these compounds are *not* substrates. These results are quite unexpected and differ dramatically from similar studies with other enzymes which presumably proceed by an analogous reaction mechanism. GAR synthetase appears to be extremely specific for glycine.

Summary. Efforts to clone and overproduce GAR synthetase have been successful in yielding large amounts of homogeneous protein. Preliminary mechanistic studies have unraveled the steady-state kinetic mechanism, and substrate specificity studies have revealed unusual properties of the glycine binding site. These studies provide the foundation for future detailed mechanistic investigations of protein binding domains involving carboxylate activation by phosphorylation. In addition, in vivo and in vitro studies are underway to investigate the possibility of direct PRA transfer between the first two enzymes in the purine biosynthetic pathway.

Registry No. PRA, 14050-66-9; GAR synthetase, 9032-01-3; MgATP, 1476-84-2; glycine, 56-40-6; DNA (*Escherichia coli* gene *pur*D), 123880-68-2; glycinamide ribonucleotide synthetase (*Escherichia coli* reduced), 123880-69-3.

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