Formyl Phosphate: A Proposed Intermediate in the Reaction Catalyzed by *Escherichia coli* PurT GAR Transformylase†

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ABSTRACT: The *Escherichia coli pur*T encoded glycinamide ribonucleotide transformylase (GAR transformylase) serves as an alternate enzyme in the production of formyl GAR for use in *de novo* purine biosynthesis. This enzyme differs from the previously known *pur*N encoded enzyme in size, sequence, and substrates; ATP and formate are required as opposed to formyl tetrahydrofolate. Kinetic studies of the wild-type PurT enzyme described here demonstrate that formyl phosphate behaves as a chemically and kinetically competent intermediate. The requirement for ATP and GAR in these reactions is consistent with previous steady-state kinetic results, which demonstrated that all substrates must be bound before catalysis. Kinetic characterization of a mutant, which releases formyl phosphate into solution, and positional isotope exchange studies also support the assignment of formyl phosphate as a plausible intermediate.

The reactions of *de novo* purine biosynthesis as originally outlined by Buchanan (1959) convert phosphoribosyl pyrophosphate to inosine monophosphate through a 10 step process. In both *Escherichia coli* and *Bacillus subtilis*, two distinct genes code for different GAR transformylases (Nygaard & Smith, 1993; Saxild et al., 1994). The *pur*N encoded transformylase utilizes a 10-formyl tetrahydrofolate cofactor (Smith et al., 1981) while the PurT transformylase requires ATP and formate as substrates as shown in eq 1 (Marolewski et al., 1994).

$$\mathrm{MgATP + HCOOH + GAR \rightarrow MgADP + PO_4^{-3} + formyl\ GAR} \quad (1)$$

The differences in substrates between these two enzymes suggest a difference in mechanisms. The PurN transformylase may proceed through a tetrahedral negatively charged transition state caused by direct attack of GAR on formyl tetrahydrofolate. A conserved water molecule in the active site serves to facilitate proton transfer during the reaction (Warren et al., 1996). Such a mechanism is unlikely for the PurT transformylase given the lower electrophilicity of formate. Chemical activation of unreactive substrates by ATP-dependent phosphorylation has been observed in several enzymes including 10-formyl tetrahydrofolate synthetase, and carbamoyl phosphate synthetase (Himes & Harmony, 1973; Wimmer et al., 1979). It was hypothesized that the PurT transformylase also functioned in a like manner, based on the overall reaction similarity to 10-formyl tetrahydrofolate synthetase and stretches of sequence homology to carbamoyl phosphate synthetase. Supporting evidence for this conjecture was the transfer of one oxygen atom from substrate formate into the inorganic phosphate product (Marolewski et al., 1994). Kinetic, mutagenic, and NMR studies presented in this paper demonstrate that the PurT reaction mechanism does proceed through a formyl phosphate intermediate.

EXPERIMENTAL PROCEDURES

Materials. β-GAR used in the kinetic assays was synthesized according to the method of Shen (1990). TX680 auxotrophic cells and the pJS455 expression vector were the kind gift of Dr. J. M. Smith. PEI cellulose TLC plates were purchased from EM Science. Sequenase 2.0 kit and restriction enzymes were obtained from United States Biochemical Co. Linker DNA, *Taq* polymerase, remaining restriction enzymes, and media were purchased from Fisher or Promega. T4 DNA ligase and nucleotides were obtained from Boehringer-Mannheim; Ultrafree-MC units were purchased from Millipore. Mutagenic primers were ordered from Integrated DNA Technologies. Radiolabeled [^{14}C]formate (specific activity = 55 mCi/mmol) was obtained from New England Nuclear, DuPont. Acetyl phosphate, carbamyl phosphate, benzoyl chloride, formic acid, $AgNO_3$, $LiClO_4$, hydroxylamine, TCA, $FeCl_3$, DTT, and KHF_2 were purchased from Aldrich. QAE A-25 Sephadex, DEAE Sephadex, HEPES, KCl, PMSF, $MgCl_2$, sodium formate, NADH, NADP, PEP, ATP, ADP, ampicillin, Ribonuclease A, pyruvate kinase/lactate dehydrogenase (700 units/mL), hexokinase/glucose-6-phosphate dehydrogenase, BSA, phenol:chloroform:isoamyl alcohol, Dalton VII molecular mass standards, inorganic phosphate determination kit, imidazole, and lysozyme were purchased from Sigma Chemical Co. Bio-Rad P60 and AG1-X4 resins were obtained from Bio-Rad laboratories. All reagents used were of the highest grade possible and were used as obtained. Kinetic measurements were made on a CARY 219 or Gilford 252 spectrophotometer. Radioactive assays were analyzed by scintillation counting using a Beckman LS8100 counter in Ecoscint fluid or quantitated on a Molecular Dynamics Phosphorimager. The FPLC

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[1] Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; BME, β-mercaptoethanol; DTT, dithiothreitol; DDF, 5,8-dideazatetrahydrofolate; GAR, glycinamide ribonucleotide; GART, GAR transformylase; fGAR, β-formyl GAR; NADH, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; PMSF, phenylmethanesulfonyl fluoride, THF, tetrahydrofolate; TLC, thin layer chromatography; PIX, positional isotope exchange.

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system and MonoQ HR10-10 column were products of Pharmacia Biotech. Electroporation was carried out in a Gene-Pulser from BioRad.

Methods

Synthesis of Formyl Phosphate. Formyl phosphate was synthesized via a two step procedure. First, formyl fluoride was prepared following the methods of Olah and Kuhn (1960). This compound was used without purification for the second step according to the procedure of Jaenicke and Koch (1963), with the modification of Smithers (1987). The final product was quantitated by trapping with hydroxylamine as detailed under detection of acyl phosphates. Phosphate impurities were analyzed separately using the method of Ames and Dubin (1960).

Detection of Acyl Phosphates. The presence of acyl phosphate was determined by conversion to the hydroxamate derivative according to the method of Pechere and Capony (1967). Briefly, the sample aliquot was incubated with an equal volume of 3 M hydroxylamine solution (at pH 7.0) for 10 min. To this was added 1 vol of 0.74 M trichloroacetic acid and 2 vol of 0.22 M $FeCl_3$ in 1 N HCl. The absorbance at 490 nm was compared to a succinohydroxamate standard for quantitation purposes.

PurT Reverse Reactions with Acyl Phosphates (Acetyl and Carbamyl). Reverse half-reactions using ADP and acyl phosphate to produce ATP were measured for both acetyl and carbamyl phosphate substrates. A coupled assay utilizing hexokinase and glucose-6-phosphate dehydrogenase was employed to monitor the production of ATP ($\epsilon = 6.22$ mM^{-1} cm^{-1} at 340 nm). Assays consisted of the following components in 1 mL cuvettes: 50 mM HEPES pH 8.0, 12 mM $MgCl_2$, 5 units of coupling enzymes, 1 mM $NADP^+$, 2.5 mM glucose, 80 nM PurT, ADP varied from 0.1−10 mM, and acyl phosphate varied from 0.25−10 mM. Each substrate, ADP and acyl phosphate, was varied at saturating concentrations of the other. Saturating concentrations of ADP were 5 mM and acyl phosphate 10 mM. Kinetic parameters were determined by fitting to the appropriate Cleland equations (Cleland, 1990).

Half-Reactions Utilizing Formyl Phosphate. Forward and reverse half-reactions utilizing the proposed intermediate were measured spectrophotometrically. For the reverse half-reaction, the production of ATP was monitored with hexokinase and glucose-6-phosphate dehydrogenase; while formyl GAR was measured using [^{14}C]GAR.

For the reverse half-reaction, three sets of assays were performed while keeping two of the three substrates at saturating levels and varying the third. Saturating concentrations of substrates were ADP 1 mM, GAR 80 μM, and formyl phosphate 10 mM. The three substrates were varied in the following concentration ranges: ADP, 0.1−1.0 mM; GAR, 5−80 μM; and formyl phosphate, 0.75−10 mM with an enzyme concentration of 43 nM in all cases. All assays were carried out in 1 mL cuvettes at 25 °C measuring initial rates at 340 nm. Kinetic parameters were determined from double reciprocal plots.

For the forward half-reaction, the conversion of formyl phosphate to formyl GAR was measured using [^{14}C]GAR. Labeled substrate and product were separated on PEI-cellulose TLC plates. Reactions consisting of 1 mM MgADP, 10 mM formyl phosphate, 10 nM PurT transformylase, 25−100 μM GAR, coupling enzymes, and reagents (5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 12 mM $MgCl_2$, 1 mM $NADP^+$, and 2.5 mM glucose) to remove ATP were incubated at 25 °C. ATP removal was necessary to prevent the production of formyl GAR from the full forward reaction of the transformylase. Aliquots were quenched by addition of HCl to bring the pH below 4. This has been shown to inactivate the enzyme without causing the hydrolysis of formyl GAR product. Time points were assayed by spotting on PEI cellulose TLC plates and developing in 50 mM potassium phosphate buffer, pH 7.0.

Mutagenesis of the PurT GAR Transformylase. Positions of mutants were determined by homology to other known enzymes, see Results for discussion. Mutant primers were designed to display a melting temperature of >50 °C and to contain a minimum number of mismatches. Primer sequences were as follows: G162I forward, 5′-CCGGTGATGAGTTCAT CCATCAAGGGGC; G162I reverse 5′-GCCCCTTGATGGATGAACTCATCACCGG. Mutagenesis was carried out by the method of PCR overlap extension (Ho et al., 1989). Nonmutagenic pUC forward and reverse primers were used along with forward and reverse mutagenic primers. Standard polymerase chain reactions were performed utilizing 1.0 μg of *pur*T wild-type template DNA, 0.5 mM dNTPs, 1× Promega PCR buffer, 2.5 mM $MgCl_2$, 1 μM forward and reverse primers, and 2.5 units of *Taq* polymerase. Denaturing temperatures of 94 °C (1 min) were followed by annealing at 52 °C (2 min) and extension at 72 °C (3.5 min) for a total of 30 cycles. PCR products were purified on a 1% agarose gel and isolated via Ultrafree-MC units. The third PCR, the overlap extension, utilized nonmutagenic outside primers, with the purified products of the first two reactions acting as templates. The third reaction was initiated at 94 °C by the addition of *Taq* polymerase (hot start PCR). After purification on agarose gel, the mutant DNA insert was ligated into the PurT expression vector overnight using standard Boehringer-Mannheim conditions. Ligated plasmid was then transformed into DH5α cells by electroporation and plated onto ampicillin containing plates (30 μg/mL).

Plasmid Purification and Sequencing of PurT G162I Mutant. Potential mutant clones were grown in liquid culture and plasmids purified via alkaline lysis and phenol extraction. Briefly, 10 mL cultures were spun down and resuspended in 250 μL of GET (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0). To this solution was added 500 μL of 0.2 M NaOH:1% SDS followed by incubation on ice for 5 min. The sample was then neutralized by the addition of 350 μL of 3 M sodium acetate, pH 5.5. This mixture was centrifuged at 14 000 rpm for 10 min at 4 °C and the supernatant transferred to a new tube. To this was added 1 mL of phenol:$CHCl_3$:isoamyl alcohol, and after vortexing briefly, the resulting suspension was centrifuged again at 14 000 rpm for 3 min at 4 °C. The aqueous layer was transferred to a fresh tube and the extraction repeated twice more. Finally, the extracted supernatant was transferred to a fresh tube and 3 mL of ethanol added. This solution was allowed to precipitate at −70 °C for 2 h before centrifugation. The purified plasmid was resuspended in 100 μL of 10 mM Tris·HCl, 1 mM EDTA, pH 8.0, and 1 μg of RNase A. Plasmid sequencing was carried out using the USB Sequenase 2.0 kit following the recommended protocols.

Complementation Assays of PurT Mutants. Mutant clones were transformed into TX680 cells using electroporation techniques following Bio-Rad protocols. The transformed cells were plated onto LB plates containing ampicillin and grown to produce single colonies. Colonies from these plates were streaked onto purine-free selective plates (Warren et al., 1996) containing ampicillin (30 μg/mL). The selective plates were incubated at 37 °C for varying lengths of time and cell growth assessed visually.

Purification of G162I PurT GAR Transformylase. Mutant enzyme was overexpressed in auxotrophic TX680 cells grown in LB at 35 °C for 12 h. Plasmid was maintained intracellularly by addition of ampicillin to a concentration of 30 μg/mL. Cells were harvested by centrifugation and lysed with the addition of 2 mg/mL lysozyme and 0.5 mg of DNase. After incubation on ice for 1 h, the lysate was cleared by centrifugation at 12 000 rpm for 25 min. The supernatant was retained and applied to a 5 × 100 cm G75 sizing column equilibrated in 50 mM HEPES, 5 mM DTT, pH 8.0. The column was run at approximately 1 mL/min with collection of 2.0 mL fractions. GAR transformylase-containing fractions were pooled prior to further purification. Purification was completed on a MonoQ HR10/10 column equilibrated in 50 mM HEPES, 5 mM DTT, 0.1 mM MgATP, pH 8.0 (buffer A). A salt gradient consisting of 0−0.5 M KCl in buffer A was used to elute the protein, with the transformylase eluting at approximately 0.1 M KCl. The purified protein, at greater than 90% homogeneity, was dialyzed to remove salt after which it was concentrated and stored frozen at −70 °C.

Measurement of ADP:Acyl Phosphate Ratio Produced by G162I PurT. Identical reactions in 50 mM HEPES, pH 8.0, containing 10 mM $MgCl_2$, 1 mM ATP, 2 mM phosphoenol pyruvate, 0.2 mM NADH, 20 units of pyruvate kinase/lactate dehydrogenase, 0.1 M hydroxylamine, varied amounts of G162I PurT transformylase, and either 100 mM acetate or 200 mM formate were incubated at room temperature for periods up to 30 min. ADP production was monitored spectrophotometrically at 340 nm. Acyl phosphate production was measured by removing aliquots and adding trichloroacetic acid and $FeCl_3$ as detailed under detection of acyl phosphates.

Synthesis of [^{18}O]Phosphate. The synthesis of this compound was carried out according to the protocol of Risley and Van Etten (1978) with the modification of Hackney (1965). [^{18}O]Enriched water containing 70% label was used as a starting material. The phosphate produced was recovered by sublimation of excess water and purified on an AG1-X4 anionic exchange column. (Hackney et al., 1965). The yield of phosphate was quantitated via the method of Ames and Dubin (1960).

Quantitation of Inorganic Phosphate. The protocol of Ames and Dubin was followed (1960) using a Sigma Phosphate Determination Kit. Briefly, 50 μL of 10% $Mg(NO_3)_2$ in ethanol was added to the sample which was then ashed over a flame. The sample was then acidified by the addition of 0.3 mL of 1 N HCl and boiled for 10−15 min to hydrolyze pyrophosphate. The boiled sample was then cooled to room temperature and 0.1 mL of 10% ascorbic acid solution added, followed by 0.7 mL of 0.42% ammonium molybdate in 1 N H_2SO_4. The solution was incubated at 37 °C for 30 min and the absorbance at 630 nm measured. A phosphoric acid standard (Sigma) was used to create a calibration curve.

Synthesis of [γ-^{18}O]ATP. Synthesis of labeled ATP was performed via the procedure of Wehrli (1965) and Middlefort and Rose (1976). The reaction was allowed to proceed at 37 °C for 24 h and the products separated on a DEAE Sephadex column as described in the author's protocol. ATP was quantitated via hexokinase/glucose-6-phosphate dehydrogenase coupled assay while impurities were checked by TLC (PEI Cellulose run in 300 mM potassium phosphate pH 7.0). The extent of ^{18}O incorporation was analyzed via NMR as described below. The samples used in this study were found to contain approximately 57% ^{18}O in the $\beta-\gamma$ bridge position. The theoretical maximum level of incorporation at this position is 70% since 70% [^{18}O]water was used as starting material.

Nuclear Magnetic Resonance Spectroscopy. ^{31}P NMR spectra for the observation of positional isotope exchange (PIX) were acquired on a Bruker Instruments AMX-500 spectrometer operating at 202.45 MHz. The sweep width was set at 7352.9 Hz with an aquisition time of 2.228 s at ambient probe temperature with broad-band proton decoupling. Total number of scans varied around 20 000 with 32 K data points. Reactions were performed in 50 mM Tris·HCl buffer (pH 8.2) containing 10 mM $MgCl_2$, 4.0 mM formate, 7.26 mM labeled ATP, and 0.4−43 μM PurT GAR transformylase at 25 °C. Aliquots of 0.5 mL were taken at a 1 h (0.4 μM enzyme) or a 3 h (43 μM enzyme) time point, quenched by addition of EDTA to a concentration of 0.5 M, extracted with $CHCl_3$ to denature the enzyme, and filtered through a Microcon-30 to insure protein removal. Samples were diluted to 1.0 mL with 99% D_2O (Aldrich) prior to data acquisition. In order to evaluate the effect of bicarbonate on the exchange process, experiments were conducted in which HCO_3^- was minimized according to the protocol of Meyer et al. (1992) with the modification of adding several freeze−pump−thaw degassing cycles prior to solution preparation. Bicarbonate concentration was determined using the PEP carboxylase method of Peled (1983). Solutions degassed in the manner described above were found to contain <20 μM bicarbonate.

RESULTS

Reactions with Acetyl and Carbamyl Phosphate. Reverse half-reaction rates were measured with these more stable formyl phosphate analogs prior to using the putative intermediate itself. The PurT transformylase is capable of generating acetyl phosphate from ATP and acetate (Marolewski et al., 1994); however, the feasibility of the reverse reaction had not been determined. Table 1 and Scheme 1 display the steady-state kinetic parameters for all PurT reactions involving acyl phosphates. Both the full- and half-reaction parameters have been included. The first entry in each division of Scheme 1 lists the complete reaction of each substrate set; cleavage of ATP to yield ADP and inorganic phosphate with concomitant production of acylated GAR. The second entry divides the complete reaction into two half-reactions. The first half-reaction leads to production of acyl phosphate, the second half-reaction acylates GAR.

Neither acetyl GAR nor carbamyl GAR is produced when the enzyme is incubated with ATP, GAR, and the appropriate acid. The full-reaction rates in Scheme 1 have therefore been

Table 1: Michaelis Constants for the PurT GAR Transformylase Reactions

substrate	complete reaction[a]	half-reactions[a]	acyl phosphate reactions[a]
full reaction[b]			
ATP	45 ± 12 μM	45 ± 12 μM	NA
GAR	10.1 ± 0.5 μM	10.1 ± 0.5 μM	32 ± 7 μM
HCOOH	319 ± 14 μM	319 ± 14 μM	NA
fp[c]	NA	NA	1.3 ± 0.2 mM
ADP	NA	NA	36 ± 10 μM
side reaction 1			
ATP	*d*	77 ± 24 μM	NA
GAR		not required	not required
CH_3COOH		3.68 ± 0.42 mM	NA
acp[c]		NA	0.26 ± 0.06 mM
ADP		NA	0.48 ± 0.02 mM
side reaction 2			
ATP	*d*	*d*	NA
GAR			not required
NH_2COOH			NA
cp[c]			1.17 ± 0.07 mM
ADP			0.49 ± 0.04 mM

[a] See text for definitions of complete and half-reactions. NA, not applicable. [b] These labels match the reactions depicted in Scheme 1. [c] Acyl phosphates have been abbreviated as follows: fp, formyl phosphate; acp, acetyl phosphate; cp, carbamyl phosphate. [d] Reaction does not occur.

set at zero to denote this fact. Neither the reverse first half-reaction with acetyl phosphate nor carbamyl phosphate requires GAR to be present, in contrast to the half-reactions involving formyl phosphate. The K_m value for ATP in side-reaction 1 (77.4 μM) compares favorably to that of the full-reaction (45 μM) suggesting that ATP is binding in the same site.

Reactions with Formyl Phosphate. Formyl phosphate was shown to be a chemically competent intermediate by separate assays in which ATP and formyl GAR production were monitored. Kinetic measurements of the reverse first half-reaction, generating ATP, were made in an identical fashion to those involving acetyl or carbamyl phosphate as described in the Experimental Procedures. These values, as well as those for the forward first half-reaction, are listed in Table 1 and Scheme 1. The half-reactions with formyl phosphate require all three substrates to be bound at the active site. Thus, the k_{cat} value for the production of ATP was measured under saturating GAR concentrations. Likewise, reactions producing formyl GAR were carried out with saturating ADP levels.

The k_{cat} for the reverse first half-reaction, 2.9 s^{-1}, closely resembles the values for the acetyl and carbamyl phosphate first half-reactions. The 32 μM K_m value for GAR is near the value for the full-reaction (10.1 μM), showing that the same binding site is probably being used. The Michaelis constant for formyl phosphate (1.3 mM) falls in the same range as those for the other acyl phosphates and suggests that the active site does not discriminate in favor of the formyl phosphate species. This value also suggests that formyl phosphate binds rather weakly in the active site. This can be understood in view of later arguments on the dissociability of this intermediate. The forward rate of the second half-reaction, producing formyl GAR from saturating formyl phosphate and ADP, was measured via radioactive assays and found to be 20 s^{-1}.

Design of G162I PurT Mutant. Limited portions of sequence homology between the PurT GAR transformylase and carbamoyl phosphate synthetase were used as a basis for designing a PurT mutant. The homology between this region of the PurT sequence and several other enzymes is illustrated in Figure 1. This glycine-rich region has been shown in porcine adenylate kinase to form a flexible loop that closes around the phosphate moieties of ATP (Egner et al., 1987). A glycine-to-isoleucine mutation at the analogous position in *E. coli* carbamoyl phosphate synthetase resulted in a 10-fold increase in the K_m for ATP and a 10-fold decrease in the rate of ATP hydrolysis (Post et al., 1990). The change to isoleucine presumably reduces the flexibility of the loop causing reduced binding affinity. It was expected that the G162I mutant would also display an increased K_m for ATP and possibly a decreased k_{cat} as well.

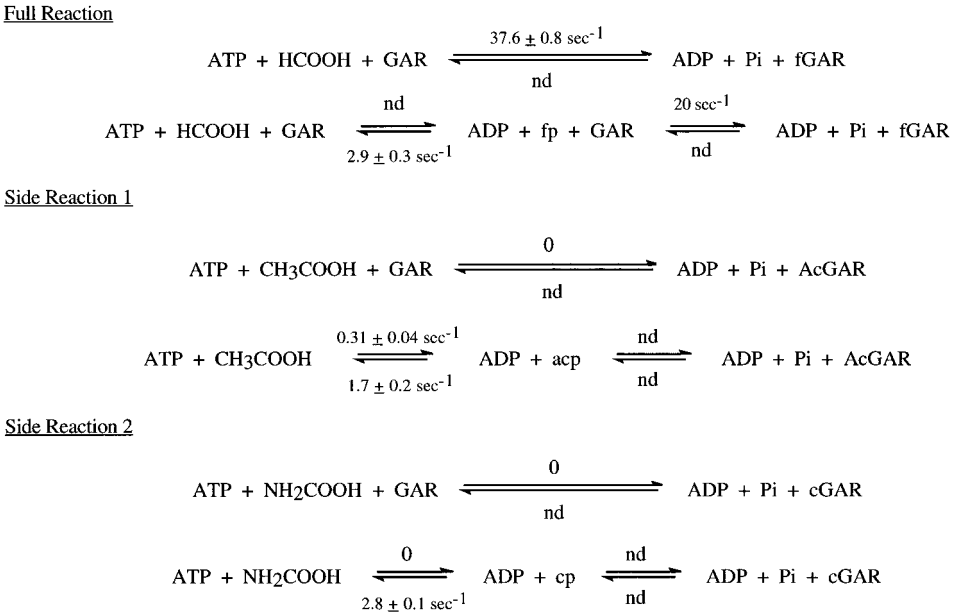
Characterization of G162I PurT. The mutant enzyme was capable of supporting auxotrophic TX680 cells grown on purine-free selective media. The rate of growth was equal to that of cells containing the wild-type transformylase, indicating that production of formyl GAR was sufficient to sustain normal rates of cell growth. G162I PurT was overexpressed and purified from auxotrophic cells in a manner identical to the wild-type enzyme. Radiolabeled assays with the purified G162I mutant, see Figure 2, confirmed the complementation results by demonstrating that formyl GAR was indeed produced by the enzyme.

Steady-state kinetic parameters for three separate reactions of the G162I PurT GAR transformylase are reported in Table 2. Only two of these three reactions are catalyzed by the wild-type enzyme. The full-reaction, producing formyl GAR, and the side-reaction, producing acetyl phosphate, have counterparts with the wild-type whereas the half-reaction does not.

The mutation initially appeared to behave as expected with the K_m for ATP altered 3-fold (134 versus 45 μM). This supports the prediction that glycine 162 is interacting with the nucleotide. The 317-fold decrease in the reaction rate for the conversion of formate to formyl GAR also parallels the trend seen in the *E. coli* carbamoyl phosphate synthetase mutation. Although the mutation was not designed to affect the GAR site, the Michaelis constant for GAR has increased 2-fold (21 μM versus 10.1 μM). However, the major difference is the dramatic increase in the K_m value for formate (53.9 versus 0.32 mM for the wild-type). Glycine 162 thus appears to be positioned to interact both with ATP and formate and mutation of this residue to isoleucine causes changes throughout the binding site. Zawadzke and co-workers have noted that a number of the enzymes possessing a glycine-rich loop motif bind acyl phosphate intermediates and suggest this site may be involved in both ATP and acyl phosphate binding (Zawadzke et al., 1991). A more complex interpretation for the increased Michaelis constant of formate is that ATP orientation has been altered, as evidenced by the 3-fold decrease in its binding affinity and that tight formate binding is dependent on interactions with both ATP and enzyme.

G162I PurT transformylase catalyzes the production of formyl GAR 2 orders of magnitude less efficiently than the wild-type enzyme. This reduced rate is apparently sufficient to sustain cell growth under limiting purine conditions. Since both the wild-type and G162I mutant protein are expressed at greater than 20% of the total cellular protein (in TX680 cells), this lack of activity may be offset by high protein

Scheme 1: Kinetic Parameters of PurT GAR Transformylase Involving Acyl Phosphates[a]



[a] Abbreviations are as follows: nd, not determined; fp, formyl phosphate; fGAR, formyl GAR; acp, acetyl phosphate; AcGAR, acetyl GAR; cp, carbamoyl phosphate; cGAR, carbamoyl GAR.

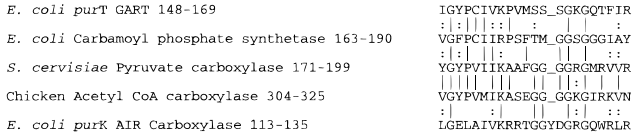


FIGURE 1: Sequence alignment of PurT residues 148−169 with various other proteins. Sequences are derived from the sources as listed; *E. coli* carbamyl phosphate synthetase (Post et al., 1990), *S. cervisiae* pyruvate carboxylase (Lim et al., 1988), chicken acetyl CoA carboxylase (Takai et al., 1988), *E. coli pur*K AIR carboxylase (Meyer et al.,1992).

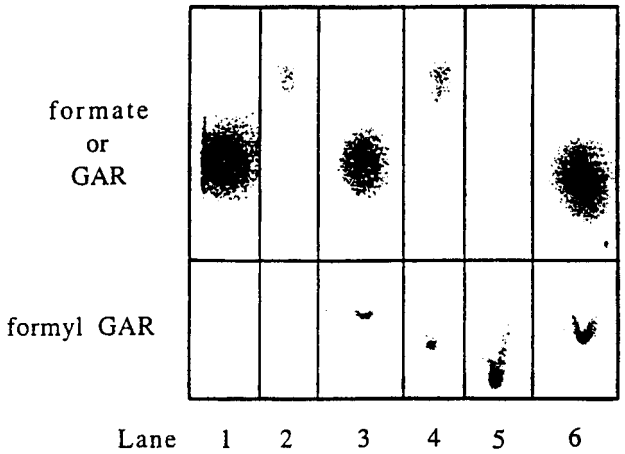


FIGURE 2: Conversion of [^{14}C]formate or [^{14}C]GAR to [^{14}C]formyl GAR by G162I PurT GART. Mutant transformylase at 15 μM concentration was incubated for 20 min with 1 mM ATP, 0.36 mM GAR, and low or high formate levels. Reaction mixtures were spotted on PEI cellulose TLC plates and developed in 50 mM phosphate buffer at pH 7.0. Lane 1, formate standard; lane 2, GAR standard; lane 3, mutant reaction with 0.56 mM labeled formate; lane 4, mutant reaction with 0.2 mM unlabeled formate and labeled GAR; lane 5, mutant reaction with 10 mM labeled formate; lane 6, reaction with labeled formate and wild-type PurT GART.

concentration. It has been previously noted that low levels of formyl GAR are sufficient for cell growth so neither protein activity nor expression of the mutant need be optimal (Smith et al., 1989).

Table 2: Reactions of G162I PurT GAR Transformylase

full reaction
$GAR + ATP + HCOO^- \rightleftharpoons fGAR + ADP + PO_4^{-3}$
half-reaction
$ATP + HCOO^- \rightleftharpoons ADP +$ formyl phosphate
side reaction
$ATP + CH_3COO^- \rightleftharpoons ADP +$ acetyl phosphate

substrate	k_{cat} (s^{-1})	K_m
full reaction		
β-GAR	0.12 ± 0.02	21 ± 4 μM
ATP		134 ± 11 μM
$HCOO^-$		54 ± 6 mM
half-reaction		
ATP	0.15 ± 0.01	99 ± 9 μM
$HCOO^-$		74 ± 11 mM
side reaction		
ATP	1.53 ± 0.04	192 ± 68 μM
CH_3COO^-		7.3 ± 1.8 mM

G162I PurT catalyzes the production of formyl phosphate from formate and ATP in the absence of GAR, which is not seen in the wild-type transformylase. The K_m for formate is moderately weaker as compared to the full-reaction, indicating that the presence of GAR strengthens formate binding. This could occur through the use of hydrogen-bonding interactions or conformational changes within the active site. The K_m for ATP has decreased very slightly for the mutant half-reaction although it has not reached the wild-type value. However, at saturating concentrations, k_{cat} values for both the half- and full-reactions are approximately equivalent (0.15 s^{-1} versus 0.12 s^{-1}). Turnover for the mutant transformylase as measured by ADP release is thus not affected by the presence of GAR, in contrast to the wild-type enzyme in which there is no release of ADP in the absence of GAR. Release of formyl phosphate as a half-reaction product was confirmed by conversion to the hydroxamate, which was visualized spectrophotometrically. The average ratio of intermediate trapped compared to ADP produced was determined to be 1.3 (±0.2) to 1.0 (±0.1).

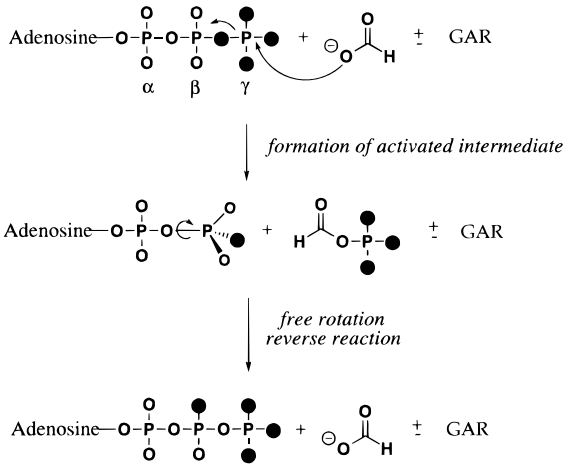


FIGURE 3: Positional isotope exchange for the PurT GAR transformylase reaction. Darkened atoms indicate ^{18}O labels.

The last reaction catalyzed by G162I PurT is the production of acetyl phosphate and ADP from acetate and ATP. The average ratio of acetyl phosphate to ADP is calculated to be 1.1 (±0.1) to 1.0 (±0.1), analogous to the ratio for wild-type transformylase. The K_m for ATP in this side-reaction is again close to the mutant K_m for the full-reaction and is higher than the wild-type value of 45 μM. G162I transformylase kinetic parameters for the side-reaction involving ATP and acetate are similar to those of the analogous reaction catalyzed by the wild-type enzyme. The Michaelis constant for acetate has only increased 2-fold from 3.68 mM in the wild-type to 7.3 mM in the mutant. The increase in K_m for ATP for this side-reaction agrees with the original hypothesis that glycine 162 is involved in ATP binding. It was expected that the 317-fold drop in the full-reaction would be reproduced in the rate of the side-reaction as well; however, the rate of this reaction has increased in the mutant to 1.5 s^{-1}.

Positional Isotope Exchange Studies. In order to obtain spectral evidence supporting the putative formyl phosphate intermediate, qualitative positional isotope exchange (PIX) experiments were conducted. Positional isotope exchange is a well-established technique for the detection of phosphorylated intermediates in enzymatic reactions involving ATP (Raushel & Villafranca, 1988). The principle of the technique as applied to the PurT GAR transformylase reaction is illustrated in Figure 3. The starting ATP is labeled in the γ-position with ^{18}O. If a phosphorylated intermediate such as formyl phosphate is produced, the ATP will be converted to ADP containing one ^{18}O label in the β-position. If free rotation of this species is permitted in the active site and there is a measurable reverse reaction rate, the label in the β-position will undergo scrambling among the $\beta-\gamma$ bridge and β-nonbridge positions in the ATP that is formed as the formyl phosphate undergoes back-reaction. The substitition of ^{18}O for ^{16}O causes a small upfield isotopic shift in the NMR resonance of the attached phosphorus. The magnitude of this shift is dependent on the P−O bond order and is approximately 0.02 ppm for a P−O single bond (Cohn & Hu, 1978). Thus, the predicted isotopic scrambling can be monitored by examining the ^{31}P NMR spectrum of the resulting ATP.

Control experiments were conducted in which enzyme was omitted in an effort to verify the lack of exchange in the absence of enzyme. As expected, no positional isotope exchange was detected in this ATP sample (Figure 4A). Labeled ATP did show positional isotope exchange in the presence of enzyme and formate (Figure 4B) and also enzyme, formate and saturating levels of GAR. Both of these results are consistent with a formyl phosphate intermediate, and the latter illustrates that the exchange rate is significantly faster than the overall reaction rate. Interestingly, positional isotope exchange was also observed when ATP was incubated with enzyme alone. This experiment yielded a spectrum showing the expected statistical distribution (a 1:2 ratio) of bridging versus nonbridging ^{18}O labels for a fully exchanged sample and was identical in appearance to that depicted in Figure 4B. This surprising finding suggested that a phosphorylated enzyme species might precede the

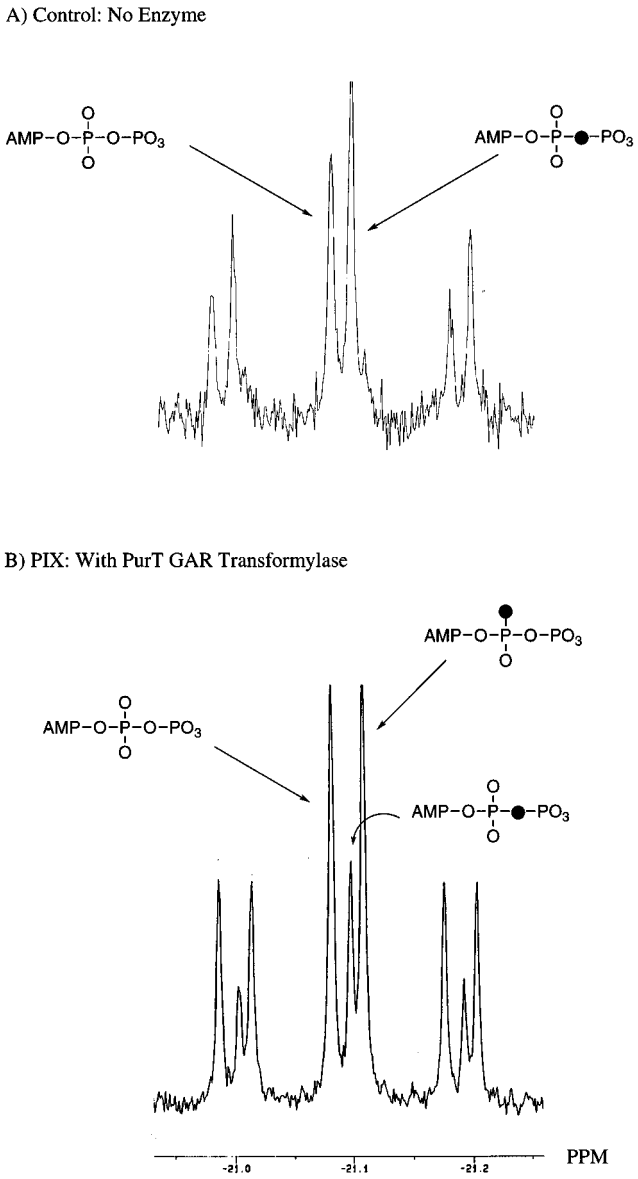


FIGURE 4: ^{31}P NMR spectra taken at 202.45 MHz of the β-phosphorus resonance of (A) ^{18}O-labeled ATP after incubation at 25 °C in the absence of enzyme and (B) ^{18}O-labeled ATP showing PIX after incubation with enzyme and formate at 25 °C as described in the Experimental Procedures. The 1:2 ratio of the upfield peaks represents the expected statistical distribution of bridging versus nonbridging ^{18}O labels. The spectrum of the PIX reaction mixture obtained after the ^{18}O-labeled ATP was treated with enzyme alone was identical in appearance to the one depicted above. Note that the intensity settings are different for spectra A and B.

formyl phosphate intermediate in the PurT GAR transformylase reaction mechanism. If such a phosphorylated enzyme species is formed, the observed PIX might be due exclusively to this intermediate instead of formyl phosphate. Alternatively, it seemed possible that the enzyme might be catalyzing nonproductive carboxyphosphate formation from the ATP and dissolved bicarbonate. Production of this species and back-reaction could also lead to the observed PIX effect.

To rule out this possiblity, the PIX experiments were repeated using solutions in which bicarbonate was minimized as described in the Experimental Procedures. Reactions using enzyme and ATP in these solutions clearly showed positional isotope exchange at a level identical to the experiments in which dissolved bicarbonate was not removed (data not shown). It should also be mentioned that bicarbonate does not serve as a substrate for the transformylase in the full-reaction or the forward half-reaction as monitored by ADP production (Marolewski et al., 1994). Thus, if the observed PIX effect were due to carboxyphosphate, formation of this substance would have to be perfectly nonproductive. This finding coupled with the NMR observations support the intermediacy of a phosphorylated enzyme prior to formyl phosphate in the PurT GAR transformylase reaction mechanism.

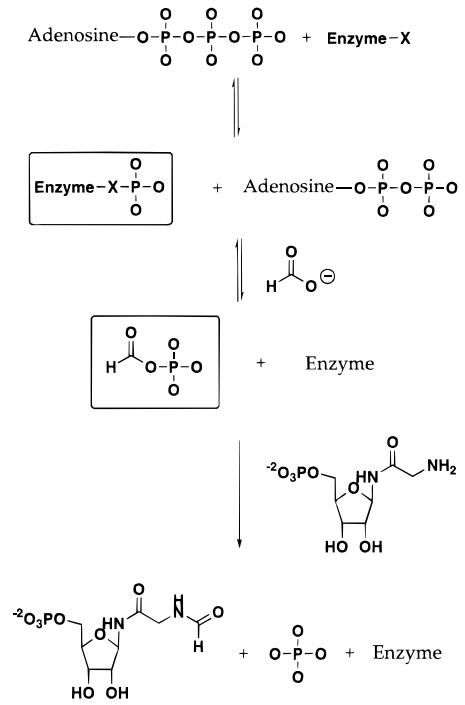


FIGURE 5: Plausible reaction mechanism for the PurT GAR transformylase.

DISCUSSION

The present experiments demonstrate that formyl phosphate functions as a substrate in both forward and reverse directions for PurT GAR transformylase. The enzyme is capable of binding formyl, acetyl, and carbamyl phosphate and catalyzing the regeneration of ATP. Since acetyl and carbamyl phosphate are larger than the putative formyl phosphate intermediate, the active site must have moderate flexibility.

The rate constants for formyl phosphate partitioning can be used to assess its kinetic competence according to the method of Cleland (1990). The putative intermediate can be classed as a nondissociable species with respect to the wild-type enzyme, although it is released into solution from the mutant active site. The ratio of intermediate dissociation to complete reaction can be calculated via eq 2:

$$k_4 = (K_{eq}'k_3K_{fp}K_{MgADP}K_{GAR})/(K_{eq}K_{HCOOH}K_{MgATP}K_{GAR}) \quad (2)$$

k_3 and k_4 denote off and on rate constants for formyl phosphate, K_x the Michaelis constant for substrate X. The equilibrium constant for production of formyl phosphate in solution, K_{eq}' has been estimated at 3×10^{-4} (Mejillano et al., 1989). The equilibrium constant for its production in the active site of formyl phosphate synthetase, K_{eq}', is thought to be 4.3 (Mejillano et al., 1989). In the absence of an experimentally determined number for the PurT transformylase and in light of the similarities between the reactions of the two enzymes, this value was set at 4.3 for the PurT transformylase as well. The other K_m values needed to solve this equation are listed in Table 1. If the turnover number of 20 s^{-1} reflects the value of k_4, then k_3 can be calculated as 0.003 s^{-1}. Formyl phosphate might then dissociate from the enzyme once in approximately 1×10^4 turnovers. This represents a sound strategy for a mechanism containing a water-labile intermediate and matches the experimental absence of formyl phosphate detected in solution from the wild-type transformylase. By requiring all three substrates to be present before creation of an unstable intermediate, the enzyme also increases the likelihood of formyl GAR production.

Mutagenesis of glycine 162 has revealed one section of the enzyme which is involved in formate and ATP binding. This residue is apparently located at the juncture of the nucleotide and acyl phosphate site, as Zawadzke and coworkers have hypothesized. Decreases in both ATP and formate binding are more consistent with this interpretation than assigning this region as purely ATP-interactive. The replacement of glycine by a large hydrophobic residue such as isoleucine could force the active site to assume a more open conformation. Such a conformation would be less optimized to catalyze the full-reaction and might leak intermediate into solution, giving rise to a measurable half-reaction rate. Release of this intermediate further confirms that the wild-type enzyme most likely proceeds through this species, although it is retained more efficiently within its active site. And lastly, the G162I mutant catalyzes the acetate and ATP reaction with similar kinetic parameters to the wild-type enzyme suggesting that acetate may open the active site of the wild-type enzyme to resemble the mutant.

Positional isotope exchange experiments are also consistent with a formyl phosphate intermediate as described above. These experiments further define the PurT GAR transformylase reaction mechanism by suggesting that a phosphorylated enzyme species is involved prior to formyl phosphate production. Experiments are currently underway to investigate this possibility further.

A plausible mechanism for the PurT GAR transformylase reaction is presented in Figure 5. It is sequential in nature wherein formyl phosphate is initially formed, followed by a nucleophilic reaction between the amine nitrogen of GAR and the formyl group of the intermediate. A proposed inital step generating a covalently linked enzyme−phosphate intermediate, prior to formyl phosphate formation, is also

consistent with current data. The PurT transformylase has evolved an active site designed to protect the high-energy intermediates formed and ensure that they are used productively for creation of formyl GAR.

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