Investigation of the Mechanism of Phosphoribosylamine Transfer from Glutamine Phosphoribosylpyrophosphate Amidotransferase to Glycinamide Ribonucleotide Synthetase†

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ABSTRACT: Phosphoribosylamine (PRA) is a product of glutamine phosphoribosylpyrophosphate amidotransferase (PRPP-AT) and a substrate for glycinamide ribonucleotide synthetase (GAR-syn), the first two enzymes in the *de novo* purine biosynthetic pathway. PRA has a half-life of 5 s under physiological conditions, hydrolyzing to ribose 5-phosphate. The instability of this purine precursor brings to question how the efficiency of transfer from one active site to the next is ensured: Is PRA transferred by free diffusion, or is it transferred directly from one enzyme to the next through a process defined as substrate channeling? Kinetic investigations of reactions containing both enzymes monitoring the appearance of the intermediate PRA and/or the product GAR were performed and compared with the predicted kinetics assuming a free diffusion mechanism of transfer. A significant discrepancy exists between the free diffusion model and the experimental data when the ratios of the two enzymes are varied. To accommodate this discrepancy, a direct transfer mechanism is proposed that is facilitated by protein−protein interactions. Experiments to provide evidence for these stable protein−protein interactions including gel chromatography, fluorescence spectroscopy, chemical cross-linking, and affinity gel chromatography; however, have all been unsuccessful. These results suggest that the requisite channeling interaction between PRPP-AT and GAR-syn, which is indicated by the kinetic results, must be a transient one.

In a metabolic sequence of reactions, the process whereby the product of one enzyme is transferred directly to the next enzyme without diffusing through the bulk solution has been defined as substrate channeling. This definition implicitly requires that substrate channeling be facilitated by specific protein−protein interactions. The importance of this phenomenon *in vivo* and *in vitro* has been addressed using a variety of experimental methods by numerous investigators over the past 30 years [recently reviewed by Ovádi (1991)]. The conclusions from many of these investigations have, however, been controversial and, in most cases, uncompelling (see *J. Theor. Biol. 152,* issue 1).

Several advantages of channeling are generally cited. The first is that metabolic rates can operate at peak efficiencies even though the average intracellular concentrations of the metabolites are well below saturating levels (Srere, 1987; Mathews, 1993). For example, channeling of dNTP precursors through a T4 DNA replication complex has been invoked to explain the much more rapid production of DNA at replication sites than can be accounted for by the measured *in vivo* concentrations of the dNTP pools (Mathews & Sinha, 1982). Further extensive studies of dNTP biosynthesis in T4 phage-infected *Escherichia coli* have provided a wealth of data in support of a channeling mechanism (Chiu & Greenberg, 1968; Chiu et al., 1982; Mathews, 1993).

A second advantage offered by channeling is protective sequestration at active sites of chemically reactive intermediates (Ovádi, 1991). Whether a chemically reactive intermediate is biologically reactive needs to be addressed in each specific case. We have chosen the purine biosynthetic pathway as a vehicle to investigate *in vitro* and *in vivo* the importance of transient protein−protein interactions and the phenomenon of substrate channeling as it applies to unstable intermediates. Specifically, the first and sixth steps in this pathway produce intermediates with half-lives of 5 s (pH 7.5, 37 °C) (see below) and 54 s (pH 7.8, 30 °C), respectively (Mueller et al., 1994) (Scheme 1). Given an *E. coli* cell with a typical intracellular enzyme concentration of 1−10 μM (Albe et al., 1990; specifically 1 μM for GAR-syn, unpublished data), a rate of intracellular free diffusion of 1×10^8 M^{-1} s^{-1} (Mastro et al., 1984) and a frequency of productive collisions between substrates and enzymes leading to catalysis of 1 in 100 (Welch, 1977), one can calculate a rate of productive interactions inside the cell of 1 s^{-1}. Given that the above values can easily vary by an order of magnitude (Welch & Easterby, 1994), the labile intermediate phosphoribosylamine (PRA)[1] with a rate constant for decomposition to ribose 5-phosphate (R-5-P) of 0.14 s^{-1} could undergo significant *in vivo* decomposition before it is productively bound to GAR-syn if the only mechanism of transfer from PRPP-AT to GAR-syn is free diffusion.

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[1] Abbreviations: GAR, glycinamide ribonucleotide; GAR-syn, GAR synthetase; PRPP, phosphoribosylpyrophosphate; PRPP-AT, PRPP amidotransferase; P_i, inorganic phosphate; AIR, aminoimidazole ribonucleotide; N^5-CAIR, *N*-carboxyaminoimidazole ribonucleotide; CAIR, 4-carboxyaminoimidazole ribonucleotide; PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase; MCCH, 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate: PRA, phosphoribosylamine; DMSO, dimethyl sulfoxide; TEA, triethylamine; SA, specific activity; TEAB, triethylammonium bicarbonate; Trp, tryptophan.

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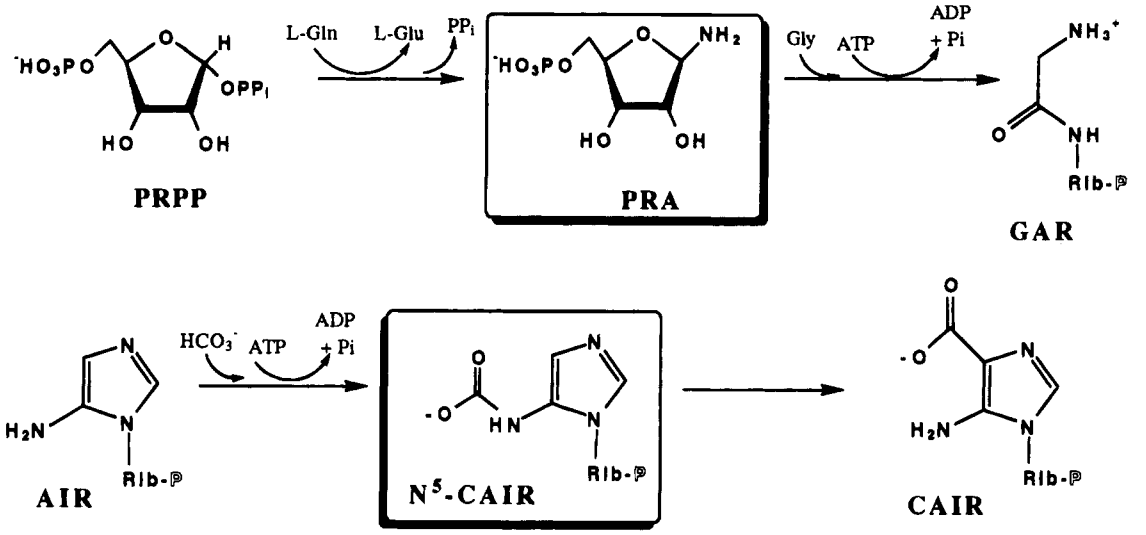
Scheme 1: (Top) First Two Reactions of the *de Novo* Purine Biosynthetic Pathway That Converts PRPP to GAR via the Unstable Intermediate PRA; (Bottom) Sixth and Seventh Reactions of the *de Novo* Purine Biosynthetic Pathway That Converts Aminoimidazole Ribonucleotide (AIR) to Carboxyaminoimidazole Ribonucleotide (CAIR) via the Unstable Intermediate N^5-CAIR



Previous extensive characterizations of phosphoribosylpyrophosphate amidotransferase (PRPP-AT) (Messenger & Zalkin, 1979; Rudolph, 1993) and glycinamide ribonucleotide synthetase (GAR-syn) (Cheng et al., 1990) have provided the basis for the present paper, which addresses the importance of direct transfer of phosphoribosylamine (PRA) between PRPP-AT and GAR-syn. Ultimately, the importance of substrate channeling needs to be demonstrated by isolation of channeling mutants. These mutants would possess an intact catalytic site and activity. However, transformation of the genes for these mutants into host cells deficient in the wild-type protein would not suppress purine auxotrophy. An *in vitro* kinetic analysis is the required first step in addressing the importance of channeling. The results described in this paper demonstrate that in the case of PRPP-AT and GAR-syn a free diffusion model does not adequately account for the observed kinetics of intermediate transfer. However, PRPP-AT and GAR-syn do not appear to form a stable isolable complex, indicating that any interaction that facilitates PRA transfer must be transient. This system thus appears to provide a good model for addressing the importance of the channeling phenomenon *in vivo*.

MATERIALS AND METHODS

Materials. NADH, ATP, bovine serum albumin (BSA), D-ribose, Sephadex DEAE-CL6B, 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester (MCCH), phosphoribosylpyrophosphate (PRPP), phosphoenolpyruvate (PEP, Na salt), molecular weight standards, pyruvate kinase (PK) (rabbit muscle, 500 μmol min^{-1} mg^{-1}) hexokinase (yeast, 280 μmol min^{-1} mg^{-1}), and lactate dehydrogenase (LDH) (rabbit muscle, 1400 μmol min^{-1} mg^{-1}) were purchased from Sigma. Acrylamide, SDS, Affi-Gel 15, Bradford assay reagent, protein A-agarose, and Dowex 50W-X8 were purchased from Bio-Rad. Succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) was obtained from Pierce. [1-^{14}C]Gly and [γ-^{32}P]ATP were obtained from New England Nuclear. Scintillation fluid (Scint-A) was obtained from Packard. All other reagents were of reagent grade.

E. coli strain pJGK10/Mi7 containing an inducible plasmid for ribokinase was a generous gift from Dr. J. N. Hope, Purdue University, Lafayette, IN. *E. coli* strain pH011/HO561 containing an overproducing plasmid for PRPP synthetase was a generous gift from Dr. R. L. Switzer, University of Illinois, Urbana, IL. *E. coli* strain pJS187/TX635 containing an overproducing vector for GAR-syn has been described (Cheng et al., 1990). *E. coli* strain pJS362/TX635 is an overproducer of PRPP-AT (J. M. Smith, Seattle Biomedical Research Institute, unpublished results). *E. coli* strain TX302 has been described (Wilson & Turnbough, 1990). BSA was used as a protein standard for all protein quantitations (A_{280} (0.1%) = 0.667). Polyclonal antibodies to PRPP-AT and GAR-syn were prepared by standard procedures and used without further purification (Johnstone & Thorpe, 1987).

Decomposition and Quantitation of PRA. PRA was synthesized from ribose 5-phosphate and ammonium hydroxide, and its rate of decomposition was measured under varying conditions essentially as described previously (Cheng et al., 1990; Schendel et al., 1988). Because of the rapid equilibrium between the α- and β-anomers of PRA, this procedure measures the total amount of PRA in solution, whereas NMR studies have shown there exists a 60:40 ratio of β-PRA to α-PRA in solution (Schendel et al., 1988).

Solution A, designed to mimic PRPP-AT/GAR-syn assay conditions, contained 100 mM Tris-HCl (pH 8.0), 6 mM Mg(OAc)$_2$, 20 mM L-Gln, 2 mM ATP, and 2 mM PRPP in a final volume of 950 μL at 18 °C. The pH of this solution was lowered to 6.0 by the addition of 50 μL of 1 N HCl such that the pH upon PRA addition was 8.0. Solution B, designed to mimic *in vivo* conditions, was prepared from a 500-mL culture of *E. coli* TX302 grown to 1.0 OD$_{600}$. The cells were harvested by centrifugation, resuspended in 2 mL of 100 mM Tris-HCl (pH 8.0), and then ruptured by passage through a French Pressure apparatus at 16 000 psi. The pH of a 950-μL aliquot of the crude extracts was lowered from 8.0 to 7.0 by addition of 25 μL of 1 N HCl such that the final pH upon PRA addition was 9.0. (Addition of HCl to below 7.0 leads to a significant amount of protein precipita-

tion.) Solution B was them preincubated at 37 °C. To measure the rate of decomposition, a 50-μL aliquot of PRA was then placed into solution A or solution B. Aliquots of 20 μL were removed from these new solutions every 15 s and placed into 80-μL quench solutions containing GAR-syn, [^{14}C]Gly, and ATP, and the [^{14}C]GAR formed was then quantitated as described previously (Schendel et al., 1988).

Isolation and Assay of PRPP-AT. PRPP-AT was isolated from pJS362/TX635 by a modification of the procedure of Messenger and Zalkin (1978). The ammonium sulfate gradient used for eluting PRPP-AT from the DEAE-CL6B column was adjusted to 0−250 mM in the buffer described by Zalkin. Further purification to homogeneity, including the removal of an ATPase activity, was achieved by Mono-Q FPLC chromatography (Pharmacia). PRPP-AT was loaded onto the column (5 × 50 mm) equilibrated in Tris-HCl buffer (50 mM, pH 8.0) at a flow rate of 1 mL/min. Following a 5-min wash of the column, a salt gradient in Tris buffer was applied over 5 min to 310 mM NaCl. Two major fractions containing protein were then eluted isocratically. The first fraction at 2.5 min into the isocratic elution contained the ATPase activity but no PRPP-AT activity. The second fraction at 7 min, which trailed considerably (up to 16 min), contained PRPP-AT activity and no ATPase activity. This second fraction was pooled and immediately concentrated and desalted by Amicon ultrafiltration (PM-30 membrane).

PRPP-AT was routinely assayed by coupling the formation of PRA to the production of GAR with a 50-fold excess of GAR-syn. The production of GAR or concomitant formation of ADP was then followed as described below.

Isolation and Assay of GAR-syn. GAR-syn was isolated as described previously (Cheng et al., 1990). The production of GAR was monitored using [^{14}C]Gly and Dowex chromatography as described (Schendel et al., 1988). Reactions could be terminated by the addition of TCA (7.5% final concentration) or by the addition of EDTA (pH 8.0, 150 mM final concentration). The formation of ADP as catalyzed by GAR-syn was monitored by using the coupling enzymes PK and LDH as previously described (Cheng et al., 1990). The V_{max} of GAR-syn (V_2) was determined by varying the concentration of PRA and fitting these activity measurements to eq 1 (Cleland, 1983):

$$v = \frac{V_{max}[S]}{K_m + [S] + [S]^2/K_i} \quad (1)$$

Kinetics Studies with Both PRPP-AT and GAR-syn. Final reaction conditions in a volume of 1 mL consisted of 100 mM Tris-HCl (pH 8.0), 20 mM L-Gln, 6.0 mM $Mg(OAc)_2$, 3.0 mM [1-^{14}C]Gly (SA = 3−5 × 10^6 cpm/μmol), 2.0 mM PRPP, 2.0 mM ATP, and variable amounts of PRPP-AT and GAR-syn. PRPP was always used to initiate the reactions following preincubation of the reaction mixtures at 18 °C for 4 min. Aliquots of 100 μL were taken at the appropriate time points and quenched into 30 μL of 30% TCA. Separation of [^{14}C]GAR and confirmation of the activity of PRPP-AT were determined as described above.

Isolation and Assay of Ribokinase and Biosynthesis of [^{32}P]-R-5-P. *E. coli* strain pJGK10/MRi7 was used to partially purify ribokinase (12-fold) (Hope et al., 1986). Ribokinase activity was monitored as described previously (Schendel et al., 1988). The isolated protein was stored at −80 °C in aliquots with 20% glycerol and retained full activty for 4 years (SA = 28.5 unit/mg). [^{32}P]R-5-P was synthesized as described for the biosynthesis of [1-^{13}C] R-5-P (Schendel et al., 1988) using [γ-^{32}P]ATP (2.0−10.0 × 10^7 cpm/μmol) and unlabeled ribose. The isolated [^{32}P]R-5-P (yield = 85%) was stored dry at −20 °C.

Isolation of PRPP Synthetase and Biosynthesis of [5′-^{32}P]-PRPP. *E. coli* strain pH011/HO561 containing an expression vector for PRPP synthetase was grown and harvested as described (Hove-Jensen, 1985). PRPP synthetase was partially purified (to 85% homogeneity) following the procedure described by Switzer and Gibson (1978). Protein purification was monitored by SDS−PAGE. Following dialysis against two changes of 50 mM KP_i (pH 6.9), the enzyme was stored at −80 °C and lost all activity over the course of 1 year.

[^{32}P]R-5-P (4.3 mM, 2.0−10.0 × 10^7 cpm/μmol) was incubated in 20 mM triethylamine−20 mM KP_i buffer (pH 7.0) with 10 mM ATP, 350 μM EDTA, 20 mM $Mg(OAc)_2$, and 4.9 mg of purified PRPP synthetase in a final volume of 2 mL. The reaction was run for 20 min at 37 °C, and the remaining ATP was converted to ADP by the addition of hexokinase (20 units) and glucose to 15 mM. After an additional 10 min, the reaction mixture was diluted into 30 mL of ice-cold distilled H_2O and loaded onto a Sephadex DEAE A-25 (8 mL) in water at 4 °C. The column was washed with 50 mL of water, and the product was eluted with a linear gradient (70 × 70 mL) of 0−900 mM TEAB (pH 7.6). Fractions were monitored for radioactivity by scintillation counting, and those containing [5′-^{32}P]PRPP, at 580-650 mM, were pooled. The TEAB was removed *in vacuo* at temperatures below 10 °C, and the [5′-^{32}P]PRPP (yield = 50−67%) was used immediately.

Product Analysis of Channeling Experiments. Kinetic experiments were performed which monitored the depletion of PRPP, the formation of PRA and its decomposition product R-5-P (both present as R-5-P following the quench), and the formation of GAR. Final reaction conditions in a volume of 1 mL consisted of 100 mM Tris-HCl (pH 8.0), 20 mM L-Gln, 6.0 mM $Mg(OAc)_2$, 3.0 mM Gly, 2.0 mM ATP, 540 μM [5′-^{32}P]PRPP (SA = 2−5 × 10^7 cpm/μmol), and variable amounts of PRPP-AT (2−20 μM/min) and GAR-syn (2−35 μM/min). Because of its instability, PRPP was always used to initiate the reaction following preincubation of the mixture at 18 °C for 4 min. A temperature of 18 °C was chosen to minimize kinetic complications arising from decomposition of PRA during the assay. At the appropriate time points, aliquots of 50 μL were quenched into 15 μL of 500 mM EDTA/100 mM Tris-HCl (pH 8.0), immediately followed by submersion in liquid N_2 and storage at −80 °C until the analysis could be performed. Control reactions were performed under the identical conditions (1) in the absence of L-Gln, (2) in the absence of Gly, or (3) in the presence of a 50−100-fold excess of GAR-syn (2000 μM/min).

Analysis of [5′-^{32}P]PRPP, [^{32}P]GAR, and [^{32}P]R-5-P was performed using ion pairing high-performance liquid chromatography (HPLC). An Alltech C18 column (4.6 × 25 cm) was equilibrated in 10 mM tetrabutylammonium hydroxide (to pH 6.0 with phosphoric acid). The flow rate was 1 mL/min, and the column was at room temperature. The samples were thawed following the addition of 350 μL

of the column buffer, and the entire solution was then injected using a 500-μL injection loop. The column was then washed with buffer for 10 min. Next, a linear gradient of methanol (0−50% v/v) was applied over 15 min. This was followed by isocratic elution for an additional 20 min. (The column was then cycled back to 0% methanol and reequilibrated for 15 min in preparation for the next injection.) The following retention times were observed: GAR, 6.0 min; unknown, 10 min; R-5-P, 18.5 min; PRPP, 29.5 min. The elution times for GAR, R-5-P, and PRPP were verified by orcinol assay using appropriate standards (Dische, 1962). The eluate was collected in 0.5-mL fractions, and the radioactivity was quantitated by scintillation counting. The amount of R-5-P formed nonenzymatically was subtracted from all experiments.

Kinetic Modeling. The Excell spreadsheet program on a Macintosh II was used to numerically integrate the differentials in eqs 2 and 3 (see Results). For an excellent introduction to modeling in spreadsheet programs, see Atkinson (1987). For analogous numerical integrations of Michaelis−Menten equations, see Duggleby et al. (1978), McClard and Shokat (1987), and Storer and Cornish-Bowden (1974). The exact differential δt was replaced with the interval Δt, which was varied in the numerical integration such that the step size was sufficiently small to accurately represent a continuous process (from 0.005 to 0.02 min). The numerical integration was separated into parts in various columns of the spreadsheet with each row containing step i. In particular, column 1 sums the time t_i using the formula $t_i = t_{(i-1)} + \Delta t$. Column 2 solves eq 2 for Δ[PRA] using $[PRA]_{(i-1)}$ and the time interval Δt. Column 3 sums the PRA concentration at time t_i using the formula $[PRA]_i = [PRA]_{(i-1)} + \Delta[PRA]$. Column 4 solves eq 3 for Δ[GAR] using $[PRA]_{(i-1)}$ and the time interval Δt. Column 5 sums the GAR concentration at time t_i using the formula $[GAR]_i = [GAR]_{(i-1)} + \Delta[GAR]$.

Gel Filtration Chromatography. The possible existence of protein−protein interactions between PRPP-AT and GAR-syn was investigated using FPLC sizing chromatography on an Altex Spherogel 3000SW and a Pharmacia Superose 12 at a flow rate of 0.5 mL/min using 50 mM Tris-HCl (pH 7.5 or pH 8.0) ± 5 mM $Mg(OAc)_2$ as the elution buffer. Molecular weight standards included carbonic anhydrase (29 000), egg albumin (45 000), GAR-syn (46 000), bovine serum albumin (66 000 and 132 000), AIR synthetase (76 000), formylglycinamide ribonucleotide amidotransferase (131 000), and LDH (140 000). Protein concentrations loaded onto the column ranged from 3 to 12 mg/mL with ratios of PRPP-AT to GAR-syn varying from 3:1 to 1:3.

Fluorescence Spectroscopy. Fluorescence spectroscopy was performed on either a Hitachi F3010 fluorescence spectrophotometer or a Perkin Elmer LS50 luminescence spectrophotometer. Protein concentrations ranged from 0.05 to 6.0 mg/mL in either 5 mM HEPES buffer (pH 7.7) or 10 mM Tris (pH 7.9). Substrates were added in various combinations to the following final concentrations: 5 mM $Mg(OAc)_2$, 10 mM L-Gln, 1 mM ATP, 2 mM PRPP, 0.1−2.0 mM PRA. All buffers and reagents (except PRA) were preequilibrated at room temperature for at least 5 min before data collection was performed. Emission spectra were collected over a wavelength range of 300−400 nm after excitation at 295 nm. The excitation bandpass and emission bandpass were set at 5 nm, the scan speed was set at 60 nm/min, and the response time was set at 2 s.

Protein Cross-Linking. In vitro cross-linking of proteins was performed as described by Ji (1983) using MCCH and SMPB. Both cross-linking reagents were suspended in 1:1 DMSO/H_2O, and the cross-linking reactions were performed in 50 mM TEA-HCl (pH 8.5). All reactions were incubated at room temperature for 0.5−2 h with protein concentrations of 1−2 mg/mL and cross-linker concentrations ranging from 0.5 to 5.0 mM. Substrates, when added, were brought to the same final concentrations as in the fluorescence experiments described above. Results were visualized by SDS−polyacrylamide gel electrophoresis (7.5% acrylamide) (Laemmli, 1970) and Western blotting (Towbin et al., 1979) with development using antibodies to either PRPP-AT or GAR-syn.

In vivo cross-linking of proteins was performed as described previously using the same cross-linking reagents as for the *in vitro* experiments (Mathews et al., 1987). *E. coli* strain TX302 was grown to 0.5 OD_{600}, harvested by centrifugation at 3000*g* at 4 °C, and resuspended in 0.01 vol of TEA-HCl (pH 8.5). A 1:1 mixture of resuspended *E. coli* and cross-linking reagents was incubated for 2 h at room temperature. The reactions were terminated by boiling with Laemmli buffer (Laemmli, 1970) and the results visualized as for the *in vitro* cross-linking reactions.

Protein Affinity Chromatography. Protein affinity chromatography was performed at 4 °C following a modification of the procedure developed by Alberts (Formosa et al., 1983). The proteins PRPP-AT, GAR-syn, and bovine serum albumin (BSA) were coupled to Affi-Gel 15 according to the Bio-Rad protocol. The column material was packed into a 1.0 × 5.0 cm column and washed with buffer containing 20 mM Tris-HCl (pH 8.0), 5 mM $Mg(OAc)_2$, 10% glycerol, and 2.0 M NaCl at a flow rate of 2−3 mL/h (LKB peristaltic pump) for 24 h. Quantitation of covalently bound protein was performed by measuring the difference between total amount of protein used for column derivatization and the amount eluted in the wash using the Bradford assay (Bio-Rad). Cell extracts were prepared from *E. coli* WT strain TX302 (grown to $OD_{600} = 0.7$ in minimal media) and subjected to affinity chromatography as described (Formosa et al., 1983). When purified PRPP-AT or GAR-syn were applied to the affinity columns, they were loaded at concentrations of 5−10 mg/mL. The eluted samples were subjected to dialyses versus distilled H_2O (30 mL vs 4 L, 4 h, 3×, 4°C) to remove salts and glycerol from the samples and then concentrated to 100 μL final volume *in vacuo*. Samples were then run on 10% SDS−PAGE (Laemmli, 1970) and visualized by either Coomassie Blue or by Western blotting with development using antibodies to PRPP-AT and/or GAR-syn (Towbin et al., 1979).

RESULTS

Kinetic Evidence for Channeling of PRA

Decomposition of PRA. An investigation of the kinetics of PRA transfer from PRPP-AT to GAR-syn requires an accurate rate constant for breakdown and anomerization of PRA. While these numbers have been previously investigated in some detail (Nierlich & Magasanik, 1965; Schendel et al., 1988), they have not been determined under the

Scheme 2: Kinetic Mechanism for Free Diffusion Transfer of PRA

$$\text{RPP} \xrightarrow{V_1} \text{PRA} \xrightarrow{K_m, V_2} \text{GAR}$$

$$\downarrow k_{decomp}$$

$$\text{R-5-P}$$

conditions used in the channeling experiments described in this paper nor under *in vivo* conditions.

The stability of PRA was therefore examined under the same conditions as those used in the lag time and product analysis experiments described below. The first-order rate constant for its decomposition, k_{decomp}, at pH 8.0 and 18 °C is 7.0×10^{-4} s^{-1}.

The stability of PRA was also investigated under conditions designed to mimic the *in vivo* environment using crude *E. coli* extracts. Unfortunately, these experiments could not be performed at pH 7.5, 37 °C. First, reducing the pH in the crude extracts by the addition of sufficient HCl so as to attain a final pH of 7.5 upon addition of the highly basic PRA solution leads to a large amount of protein precipitation. Second, attempts to use standard rapid quench technology required to measure a rate constant of 0.14 s^{-1} failed due to our inability to quench the reaction without the destruction of PRA. The rate of PRA decomposition was therefore determined at pH 9.0 and 37 °C to be 0.014 s^{-1}. On the basis of the pH dependence of this decomposition described previously, this number could be extrapolated to 0.14 s^{-1} at pH 7.5 (Schendel et al., 1988).

Description of Free Diffusion Model. The kinetics of PRA transfer from PRPP-AT to GAR-syn can be described by Scheme 2 and eqs 2 and 3, if the experiments are carried out under appropriate conditions.

$$\frac{\delta[\text{PRA}]}{\delta t} = V_1 - \frac{V_2[\text{PRA}]}{K_{m(\text{PRA})} + [\text{PRA}]} - k_{decomp}[\text{PRA}] \quad (2)$$

$$\frac{\delta[\text{GAR}]}{\delta t} = \frac{V_2[\text{PRA}]}{K_{m(\text{PRA})} + [\text{PRA}]} \quad (3)$$

V_1 and V_2 are the amounts of PRPP-AT and GAR-syn (in units of μM/min), respectively. $K_{m(PRA)}$ is the K_m of PRA for GAR-syn. PRA is formed by PRPP-AT at a fixed rate (V_1) using saturating concentrations of substrates PRPP and L-Gln. PRA is removed by decomposition to R-5-P at a fixed rate (k_{decomp}) and by conversion to GAR by GAR-syn, where this interaction is governed by the Michaelis–Menten equation for the substrate PRA when the other substrates (Gly and ATP) are saturating.

In addition to using saturating concentrations of substrates, the reaction is quenched prior to producing concentrations of products that will lead to product inhibition and where the GAR-syn reaction is essentially irreversible. For example, the buildup of the most potent inhibitor ADP (K_i = 245 μM; Cheng et al., 1990) to a concentration of 70 μM leads to a 2% reduction in V_2 given a 2 mM concentration of ATP. Also, given the formation of 70 μM of each of the products (P_i, ADP, GAR), the rate of GAR-syn in the reverse direction would be <1% of V_{max} in the forward direction based on the appropriate form of the terreactant equation and the product inhibition by PRA observed in the reverse reaction (Cheng et al., 1990).

In Scheme 2 and eqs 2 and 3, the four key parameters that need to be experimentally determined for simulating the free diffusion kinetics of PRA are as follows: V_1, $K_{m(PRA)}$, V_2, and k_{decomp}. Numerical values for each of these parameters were determined under conditions (pH 8.0, 18 °C) chosen to investigate the question of channeling in this two-enzyme system. These conditions were chosen to avoid complications in the kinetic analysis caused by the decomposition of PRA (Cheng et al., 1990).

V_1 was determined by measurement of the specific activity of PRPP-AT using the continuous spectrophotometric assay coupled to GAR-syn, PK, and LDH prior to each experiment. The specific activity of PRPP-AT at 18 °C was 8.1 ± 1.1 μmol min^{-1} mg^{-1} (±14%, 21 determinations). The $K_{m(PRA)}$ for GAR-syn was redetermined to be 98 ±12 μM (±12%, five determinations) by variation of the PRA concentration from 7 to 300 μM. These redeterminations also brought to light the fact that PRA exhibits substrate inhibition, an observation which was not reported previously (Cheng et al., 1990). Reevaluation of the kinetic data using eq 1 describing substrate inhibition gave a $K_{m(PRA)}$ identical within experimental error to that determined above and a K_i for PRA of 670 ± 50 μM. Assuming that β-PRA is the actual substrate for GAR-syn and given that there exists a 60:40 mixture of β-PRA to α-PRA, the actual K_m for β-PRA is 64 μM.[2] V_2 was determined using eq 1 to fit the results from the measurement of the specific activity of GAR-syn using the spectrophotometric assay at varying concentrations of PRA (5–8 different concentrations) (Cleland, 1983). The specific activity of GAR-syn at 18 °C was 42.5 ± 2.5 μmol min^{-1} mg^{-1} (±6%, 18 determinations). The rate of PRA hydrolysis, k_{decomp}, under the conditions used in the channeling experiments was found to be 0.0050 ± 0.0006 min^{-1} (±12%, 2 determinations), as described above.

Given the experimental determination of the parameters V_1, K_m, V_2, and k_{decomp}, eqs 2 and 3 can be numerically integrated as described in Materials and Methods. These simulations of the formation of (PRA and R-5-P) and/or GAR can then be used in comparisons of the free diffusion model with the experimental data obtained in the lag time and product analysis experiments described below. The model can also be easily used for error analysis by variations in any or all of the input parameters.

Lag Time Experiments and Comparison with Free Diffusion Model

In order to investigate the mechanism of transfer of PRA from PRPP-AT to GAR-syn, the coupled reaction between PRPP-AT and GAR-syn was monitored by following the progress curves for [^{14}C]GAR formation. In numerous experiments, the PRPP-AT:GAR-syn ratio was varied from 2:1 to 1:6 (V_1:V_2). If PRA proceeds from one active site to the other by free diffusion with concomitant equilibration

[2] The extremely rapid anomerization rate (3.6 s^{-1} at pD 8.5, 15 °C; Schendel et al., 1988) has prevented not only the experimental determination of the actual substrate for GAR-syn (β-PRA vs α-PRA) but has also made studies of the potential inhibition by one or the other impossible. However, the rapid anomerization also ensures that the 60:40 ratio of β:α exists under all experimental conditions and that any potential inhibition would be included in the apparent K_m, which was determined as described.

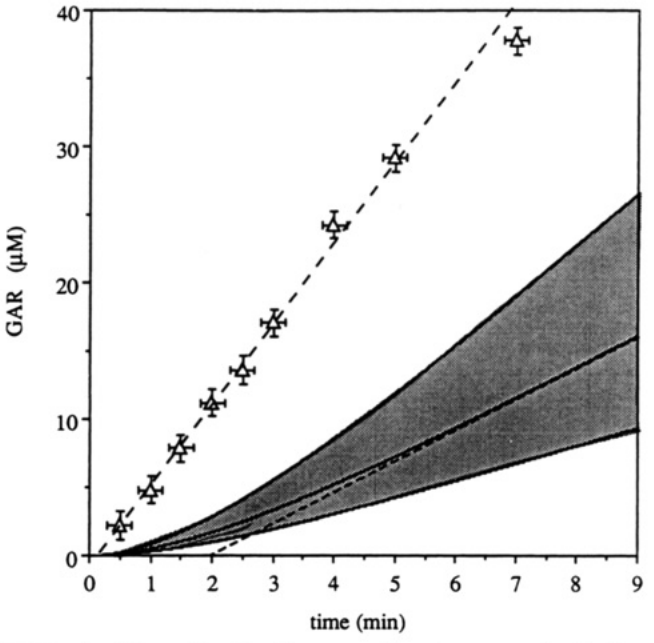


FIGURE 1: Channeling lag time experiment compared with free diffusion model using 10.5 μM/min PRPP-AT and 11.4 μM/min GAR-syn: (△) observed GAR, (—) predicted GAR from free diffusion model, and (— —) back-extrapolated lines from apparent linearity to demonstrate lag times. The shaded area indicates the predicted variation in GAR formation from the free diffusion model allowing for a 20% simultaneous error in the parameters V_1, V_2, k_{decomp}, and K_m.

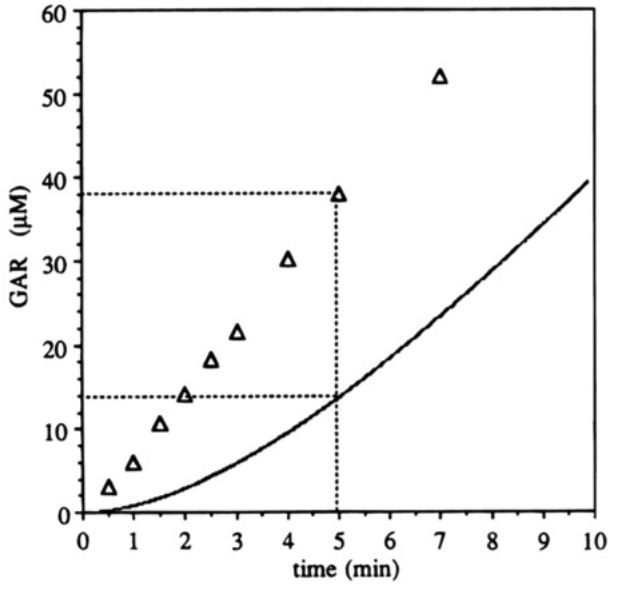


FIGURE 2: Channeling lag time experiment compared with free diffusion model using 10.5 μM/min PRPP-AT and 17.0 μM/min GAR-syn: (△) observed GAR and (—) predicted GAR from free diffusion model.

in the assay mixture, one would expect to see an apparent lag time in GAR formation as seen for the simulated progress curves in Figures 1 and 2. In all cases examined, the observed results did not agree with the simulated progress curves for the modeled free diffusion kinetics. As shown in the typical examples with PRPP-AT:GAR-syn ratios of 1:1 and 1:1.7 in Figures 1 and 2, there exists little or no apparent lag time in the production of GAR, in distinct contrast with the model. In Figure 1, for example, the apparent lag determined by back-extrapolation of the experimental data is 0.1 min, whereas the apparent lag time for the data generated from the free diffusion model is 2.1 min. Another way of exemplifying the magnitude of the discrepancy is shown in Figure 2. At 5 min, the model predicts that only 14 μM of GAR should have formed, yet 38 μM is actually observed. The control experiments performed with a 50−100-fold excess of GAR-syn showed the expected amount of PRPP-AT activity (±10%) without any observable lag time (data not shown).

The observed discrepancy between the free diffusion model and the experimental data cannot be explained by accumulated errors in the parameters used to model free diffusion. Simultaneous variation of the model parameters V_1, V_2, K_m, and k_{decomp} up to a generous error margin of 20% (see above) does not lead to an adequate fit of the modeled curve for GAR formation with the experimental data (Figure 1). This demonstrates that the experimental results obtained are incompatible with a free diffusion mechanism of PRA transfer, and thus the existence of an alternate mechanism is required.

Product Analysis Channeling Experiments. Experimental methods were developed to simultaneously monitor depletion of PRPP, the formation GAR, and the breakdown of PRA to R-5-P. To carry out these experiments, the substrate [5′-^{32}P]PRPP was enzymatically prepared from ribose and [γ-^{32}P]ATP in two steps using ribokinase and PRPP synthetase. [5′-^{32}P]PRPP could be obtained in 98% radiochemical purity by this method (HPLC analysis). Unfortunately, the instability of PRA and PRPP limit the options for quenching and analysis. For example, quenching the reactions in concentrated base (e.g., 0.2−0.5 M KOH), where PRA has a half-life of >1 h, followed by analysis under basic conditions (e.g., Dionex HPLC columns) was not feasible because of the instability of PRPP under these same conditions. HPLC analysis of [^{32}P]PRPP showed that 10−25% of the [^{32}P]PRPP had decomposed after 5−20 min at pH 14. Control quench experiments performed under a variety of acidic conditions (e.g., 3−10% TCA, 0.3−3% $HClO_4$) also showed significant hydrolysis of [^{32}P]PRPP to [^{32}P]R-5-P (5−15%) and an additional unknown (5−15%). Therefore, quenching with 150 mM EDTA (pH 8.0) (final concentration) was used, as it had previously been demonstrated to stop both the PRPP-AT and GAR-syn reactions in control experiments. PRPP was stable under these conditions, whereas PRA was quantitatively hydrolyzed to R-5-P. [^{32}P]PRPP could then be separated from [^{32}P]GAR and [^{32}P]R-5-P by HPLC (Figure 3). The [^{32}P]R-5-P detected arises from four sources: breakdown of PRPP during the reaction, breakdown of PRA during the reaction, breakdown of PRPP during the quench, and breakdown of PRA during the quench.

Three essential control experiments were performed and analyzed that allowed the interpretation of the actual experiments. First, a complete reaction mixture lacking only L-Gln was analyzed in order to monitor nonenzymatic decomposition of [^{32}P]PRPP. At temperatures greater than 18 °C, the nonenzymatic decomposition of PRPP to R-5-P and an unknown ^{32}P-labeled compound (perhaps the 1′, 2′-cyclic, 5′-diphosphate (McClard et al., 1984)) occurs at a rate fast enough to form large amounts of [^{32}P]R-5-P, which interfere in the analysis of the amount of PRA formed during the PRPP-AT catalyzed reaction. Under the experimental conditions described above, nonenzymatic hydrolysis of [^{32}P]PRPP to [^{32}P]R-5-P was found to be independent of all enzyme

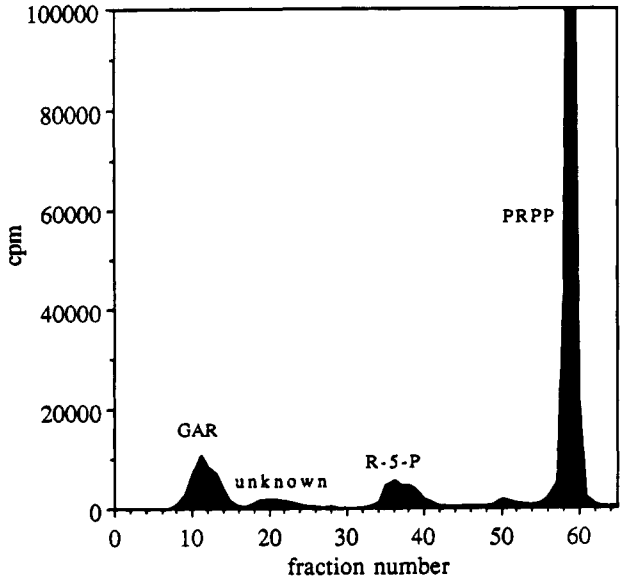


FIGURE 3: Representative HPLC trace from channeling product analysis experiments. GAR elutes at 6.0 min, the unknown elutes at 10 min, R-5-P elutes at 18.5 min, and PRPP elutes at 29.5 min.

concentrations and constant at 0.0017 min^{-1} (e.g., Figure 5). In addition, nonenzymatic decomposition of [^{32}P]PRPP to the unknown ^{32}P-labeled compound was found to be independent of all enzyme concentrations and constant at 0.0019 min^{-1}.

The second control, a complete reaction mixture lacking only Gly, was analyzed to check the activity of PRPP-AT by measurement of the amount of [^{32}P]R-5-P produced. The observed rate of [^{32}P]R-5-P formation correlated well with the expected V_1 based on the coupled assay described (±7%) and confirmed the efficacy of the EDTA quench for the PRPP-AT reaction. This control also confirms that no other ^{32}P-labeled product is produced which comigrates with GAR during the analysis by HPLC.

The third control, a complete reaction mixture containing a 50−100-fold excess of GAR-syn over PRPP-AT, was analyzed to check the activity of PRPP-AT by the amount of [^{32}P]GAR produced. The observed rate of [^{32}P]GAR formation correlated well with the expected V_1 as determined by the coupled spectrophotometric assay monitoring ADP formation (±10%) (Cheng et al., 1990). This control also confirms that the rate of [^{32}P]PRPP conversion to [^{32}P]PRA by PRPP-AT is independent of the GAR-syn concentration. This latter result also supports the correlation of the glutamate dehydrogenase and the GAR-syn coupled assays for PRPP-AT (Rudolph, 1993).

With the controls giving satisfactory results, a series of product analysis experiments were then performed at varying ratios of PRPP-AT:GAR-syn (1:0.9 to 1:4) in the nanomolar range for overall enzyme concentrations. The results were then compared with the progress curves for R-5-P and GAR formation predicted by the free diffusion model. Figures 4 and 5 show representative results of these experiments. As seen previously in the lag time studies (Figures 1 and 2), the experimental data fail to fit the curves simulated for a free diffusion mode of transfer of PRA from PRPP-AT to GAR-syn. The maximum amount of PRA and R-5-P formed is substantially below the amount predicted for a free diffusion mechanism of transfer. Also, the short (<0.3 min)

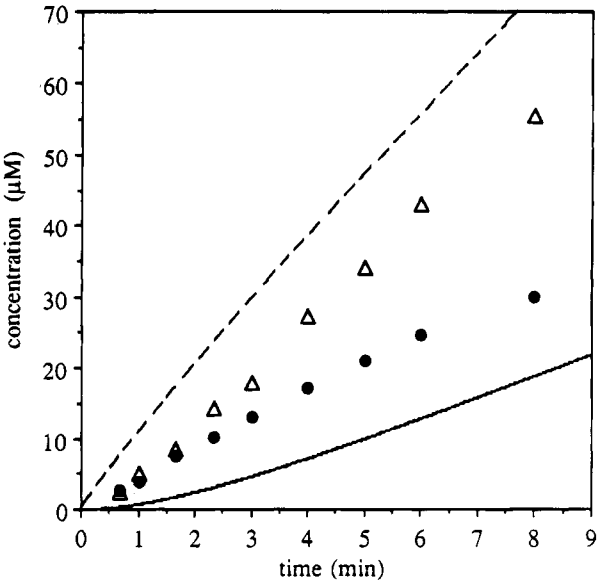


FIGURE 4: Channeling product analysis experiment compared with Excell free diffusion model using 11.4 μM/min PRPP-AT and 15.0 μM/min GAR-syn: (△) observed GAR, (●) observed R-5-P, (—) predicted GAR from free diffusion model, and (− −) predicted R-5-P from free diffusion model. The amount of R-5-P arising from the nonenzymatic decomposition of PRPP has been subtracted from the values shown.

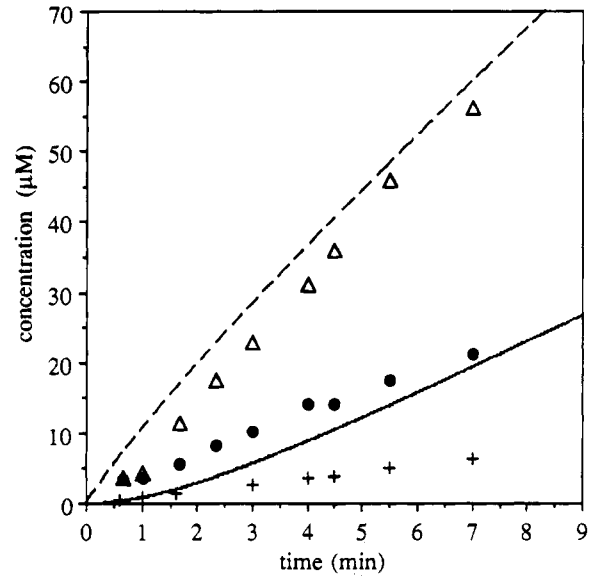


FIGURE 5: Channeling product analysis experiment compared with Excell free diffusion model using 11.4 μM/min PRPP-AT and 20.0 μM/min GAR-syn: (△) observed GAR, (●) observed R-5-P, (—) predicted GAR from free diffusion model, and (− −) predicted R-5-P from free diffusion model. The amount of R-5-P arising from the nonenzymatic decomposition of PRPP is shown (+) and has been subtracted from the amount of R-5-P shown (●).

lag times observed in the production of GAR are significantly different from thoe predicted by the free diffusion model.

Variations in the model parameters V_1, V_2, $K_{m(PRA)}$, and k_{decomp} within their experimental errors did not lead to an agreement of the model with the experimental data. An acceptable superposition of the simulated curves with the experimental data was obtained only after dramatic alterations in the input parameters for the model: The K_m of PRA needed to be reduced from 98 to 0.9−5 μM with concurrent reduction of V_2 by factors of 2−4. Even in this case, the fit to the early time points is poor with respect to the model

(data not shown). The reduction in the K_m by up to 2 orders of magnitude and the decrease in V_2 by factors of 2−5 are significantly outside the observed variability in these values (6−12%). In addition, the adjustments required to obtain possible fits varied randomly from experiment to experiment. Therefore, these product analysis experiments and their comparison to the free diffusion model provide convincing evidence that the transfer of PRA from PRPP-AT to GAR-syn does not occur by free diffusion alone.

Efforts To Obtain Evidence for Protein−Protein Interactions

An alternative to the free diffusion model could be one involving the direct transfer of PRA between PRPP-AT and GAR syn. The K_d for this protein−protein interaction would be expected to be in the nanomolar range, given the nanomolar concentrations of PRPP-AT and GAR-syn used in the experiments described above. Such a high affinity should be easily detectable by a variety of techniques. However, it is important to consider that this interaction may require the presence of the intermediate PRA and may be transient. Many of the methods traditionally used in studies of protein−protein interactions are not cmopatible with the short half-life of PRA, nor are they conducive to the detection of transient protein−protein interactions. Therefore a wide variety of experimental techniques with reaction mixtures containing both proteins in the presence and absence of PRA were undertaken to look for evidence in support of the proposed channeling interaction indicated by the kinetic results.

Size Exclusion Chromatography. FPLC gel filtration size exclusion chromatography was performed in search of a possible stable protein−protein interaction between PRPP-AT and GAR-syn. PRPP-AT alone was seen to migrate as a dimer with an apparent molecular weight of 130 000. This result contrasts with those previously reported using size exclusion chromatography, that PRPP-AT is a tetramer (Messenger & Zalkin, 1978). GAR-syn alone migrated as a monomer of molecular weight 47 000. When PRPP-AT and GAR-syn were applied together to the sizing column after preincubation at approximately equal concentrations (60−240 μM), no new higher molecular weight species were detected (data not shown). Given the concentrations of proteins applied to the column and the 20−50-fold dilution through the column, a stable interaction with a K_d as high as 5 μM should have been detectable. Therefore, if PRPP-AT and GAR-syn form a stable complex with one another in the absence of the intermediate PRA, the K_d must be greater than 5 μM. If, however, the actual channeling interaction is transient or exists only in the presence of PRA, then it would not be detected by this method.

Fluorescence Spectroscopy. An interaction between PRPP-AT and GAR-syn has the potential of causing a detectable change in the intrinsic tryptophan (Trp) fluorescence spectrum resulting from a change in the Trp environment in one or both of the proteins. The formation of a putative 1:1 complex between PRPP-AT and GAR-syn potentially can cause a change in the environment of any number of the seven Trp residues present (PRPP-AT contains two Trp, GAR-syn contains five Trp). Experiments were performed at 1−3 μM of the two enzymes where the inner filter effect was shown to be negligible (Stryer, 1968). The emission spectrum obtained upon mixing of PRPP-AT and GAR-syn was the sum of the individual spectra. No quenching, enhancement, or shift of the emission maximum was observed (data not shown). Experiments performed at concentrations of protein from 10 to 120 μM displayed no shift (±2 nm) in the fluorescence maximum upon mixing of the two enzymes. Additionally, there was no change observed after the addition of various combinations of the substrates PRPP, PRA, L-Gln, and ATP at either low or high concentrations of one or both of the enzymes. As no change in the intrinsic Trp fluorescence was observed in any of the experiments, these results have no predilection in regard to an interaction between PRPP-AT and GAR-syn.

Cross-Linking. In vitro cross-linking experiments were performed using mixtures of purified PRPP-AT and GAR-syn (20−40 μM) in the presence and absence of various substrates, including PRA. The cross-linking reagents MCCH and SMPB effected efficient (95%) dimerization of PRPP-AT, independent of the presence of the substrates L-Gln and PRPP, the product PRA, or GAR-syn (data not shown). These same two reagents gave no cross-linked products between PRPP-AT and GAR-syn as visualized by Coomassie-stained SDS−PAGE and/or Western blotting using antibodies to both PRPP-AT and GAR-syn, regardless of the presence of various substrates and/or products (data not shown).

In vivo cross-linking experiments were performed in concentrated (OD_{600} = 25) suspensions of intact *E. coli* with the reagents MCCH and SMPB as described (Mathews et al., 1987). PRPP-AT was found in its dimerized form in reasonable yield (20−50%) as estimated by Western blotting using antibodies against PRPP-AT (data not shown). As with *in vitro* cross-linking experiments, however, no cross-linked products which reacted with antibodies to both PRPP-AT and GAR-syn were found at any molecular weight or at the interface between the stacking and the running gel.

The observed cross-linking of PRPP-AT to its dimeric form in both the *in vitro* and *in vivo* experiments is an indication that the cross-linking reagents MCCH and SMPB were working under the experimental conditions. These results also contrast with previous studies of Messenger and Zalkin (1978) in which monomeric, dimeric, and trimeric species were observed using the cross-linking agent dimethyl dodecane diimidate. The fact that no cross-linking of PRPP-AT and GAR-syn was observed may be due to the reported slow cross-linking reaction in comparison with the potentially transient interaction between the two proteins (Ji, 1983). In summary, the cross-linking experiments did not allow the detection of a stable complex of PRPP-AT and GAR-syn *in vitro* or *in vivo*, even in the presence of the intermediate PRA.

Protein Affinity Chromatography. Protein affinity chromatography was employed in search of evidence of protein−protein interactions between PRPP-AT and GAR-syn. This technique, pioneered by Alberts (Formosa et al., 1983), involves the immobilization of protein onto a column, thereby creating an affinity column with a high concentration of potential binding sites for a metabolic partner. By passing either crude extracts or purified proteins over such a column, selective retention of proteins has been observed for proteins with itneractions having K_d's between 10 nM (Corin et al., 1991) and 1 mM (Zopf & Ohlson, 1990).

PRPP-AT, GAR-syn, and BSA (as a control) were used to create protein affinity columns. In all cases, high concentrations of protein covalently attached to the resin were achieved (5, 22, and 15 mg/mL, respectively). Application of crude extracts of *E. coli* to each of the columns, followed by elution with increasing concentrations of salt, led to variable results. No one protein was consistently retained, and of those proteins which were selectively held on the columns, none were observed to react with the antibodies to PRPP-AT or GAR-syn. In addition, application of purified PRPP-AT and GAR-syn to each of the columns showed no selective retention of either protein on any of the columns. Given concentrations of protein bound to the columns (>100 μM), one would have expected to detect binding if the K_d for a putative PRPP-AT−GAR-syn complex was of a comparable strength or tighter.

There are two explanations for these inconclusive results. First, the process of attaching the proteins to the column material may have altered the structure of the proteins to a non-native state. The covalently bound protein may not be oriented correctly so as to allow protein−protein interactions. Problems could especially exist with PRPP-AT. IF PRPP-AT is bound to the column as a monomer, but its native dimer form is required for an interaction with GAR-syn, then no complex would be observed. Second, the conditions for complex formation may have been inadequate due to the absence of PRA, as its short half-life is not compatible with the long chromatography times (days). A stable carbocyclic analogue of PRA has been synthesized (Caperelli & Liu, 1991). If it exhibits suitable binding properties to PRPP-AT and/or GAR-syn, it could potentially be successful in promoting an interaction between the two proteins by this technique or any of the other techniques described above.

DISCUSSION

Given that the kinetic data do not match (Figures 1−5) the progress curves for intermediate and product formation predicted by a free diffusion mode of PRA transfer, an alternative mode of transfer is required. As mentioned in the introduction, the physical concept which provides the basis for the derivation of an alternative kinetic model is the phenomenon known as channeling, the direct transfer of the intermediate from one enzyme to the other through a specific protein−protein interaction (Srivastava & Bernhard, 1986). How this channeling phenomenon can be described mathematically and then tested experimentally is a difficult question that has not been adequately resolved. A "reduced volume" model (Reddy et al., 1977; Spivey & Merz, 1989), which assumes a greatly reduced K_m of a PRPP-AT·PRA complex for GAR-syn is one possibility. Another is the partitioning of PRA between release into solution from PRPP-AT and direct transfer to GAR-syn. Either of these types of interactions would be expected to be governed by a binding constant for the PRPP-AT·PRA·GAR-syn interaction. Given the deviations in the kinetics of PRA transfer under experimental conditions using nanomolar enzyme concentrations that have been observed, such an affinity constant must also be in the nanomolar range. Yet, all of the biophysical methods applied to find evidence of a PRPP-AT·GAR-syn (·PRA) complex have been unsuccessful. This implies that the interaction between PRPP-AT and GAR-syn, which is suggested by the kinetic results, must be a transient one, most likely governed by the presence of product/substrate, PRA. This is not a surprising observation as truly efficient channeling would be unlikely to involve formation of stable observable complexes.

Channeling, as facilitated by a transient protein−protein interaction, is unlike the two structurally characterized examples of channeling, tryptophan synthase and thymidylate synthase−dihydrofolate reductase. In the bifunctional enzyme tryptophan synthase, a 25-Å hydrophobic tunnel facilitates the transfer of the uncharged indole intermediate from one active site to the other in the stable complex (Hyde et al., 1988). The recent structure determination of the bifunctional thymidylate synthase−dihydrofolate reductase indicates that the unusual surface charge distribution may play a role in channeling the intermediate dihydrofolate (Knighton et al., 1994). Both of these examples consist of a stable (or covalent) and isolable complex and bring to question whether they should be classified as channeling or as enzymatic reaction intermediates (Knowles, 1991).

It has been suggested that transient protein interactions play the key roles in biochemistry [e.g., McLendon et al. (1991) and Williams (1991)]. Such interactions are relatively uncharacterized phenomena, yet they are not without precedent. Convincing evidence for their existence has been reported in a number of systems, although only transient fluorescence spectroscopy has been developed thus far for their study. A few important examples are highlighted below.

Cytochrome *c* and cytochrome *c* peroxidase have been extensively studied in regard to the protein−protein interaction that must occur during electron transfer from one to the other (McLendon, 1988). The recent crystal structure of a cytochrome *c*−cytochrome *c* peroxidase complex has suggested a specific interaction between the two proteins with the formation of a novel pathway for electron transfer (Pelletier & Kraut, 1992). Electron transfer occurs extremely rapidly (10^3−10^4 s^{-1}) and with great efficiency (>99%). Yet, stable protein complexes can only be detected using fluorescence spectroscopy at low ionic strengths (10−20 mM) (Vitello & Erman, 1987). The presence of physiological salt concentrations (≈200 mM) does not allow the detection of protein−protein interactions even though the rate of electron transfer measured under these conditions is found to be faster than under the low salt conditions where a complex can be isolated (McLendon, 1988). Under these conditions, the binding must be strong enough to ensure that the complex lasts long enough to permit electron transfer (about 10^{-3} s for cytochrome *c* and cytochrome *c* peroxidase).

A second example of an imperative protein−protein interaction for which no evidence exists is the case of the *E. coli* ribonucleotide reductase and one of its reducing partners, thioredoxin (Holmgren, 1989). Ribonucleotide reductase catalyzes the conversion of nucleotides to deoxynucleotides concomitant with oxidation of two cysteines in the active site to a disulfide. Re-reduction of the disulfide via thioredoxin by disulfide interchange are required to regenerate active reductase (Holmgren, 1985). *In vivo* and *in vitro* cross-linking experiments, however, at 5 μM protein concentrations have been unsuccessful in providing evidence for formation of a complex between these two proteins (Mathews et al., 1987).

A third example is provided by the studies of Roux and Walsh (1992) on *p*-aminobenzoate (PABA) synthase, where protein−protein interactions must occur yet no evidence for

complex formation exists. The synthase requires three gene products (PabA, PabB, and PabC). PabB encodes the chorismate aminating enzyme. PabC is required for the elimination of pyruvate and the aromatization to yield PABA, and PabA is a glutaminase providing the "NH_3" equivalents required for PabB. PabA, however, has no catalytic activity until PabB is added. Maximal glutaminase activity is observed when a stoichiometric amount of PabB is added to PabA. The dissociation constant between the two proteins was estimated to be less than 10 nM by sequential dilution of a 1:1 mixture of the enzymes and measurement of the activation of glutaminase activity. Size exclusion gel chromatography at micromolar concentrations of PabA and PabB, however, gave no evidence for complex formation between the two proteins. The chemistry of PABA synthase requires protein−protein interactions. No interaction has thus far been detected, suggesting it must be transient.

These three examples provide precedents for systems where highly specific protein−protein interactions must occur and yet no stable protein complex can be detected or isolated. These interactions could be transient, with half-lives on the order of milliseconds. The results presented in this paper provide evidence that channeling of PRA between PRPP-AT and GAR-syn is another example of an important process that is facilitated by such a transient protein−protein interaction. Random and site-directed mutants of PRPP-AT, based on the recent X-ray structure of the *Bacillus subtilis* AT (Smith et al., 1994), will be generated in an effort to find "channeling mutants", which can then be examined in *E. coli* strains auxotrophic for purines and potentially provide evidence for the existence of channeling *in vivo*.

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