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Characterization and Chemical Properties of Phosphoribosylamine, an Unstable Intermediate in the de Novo Purine Biosynthetic Pathway†

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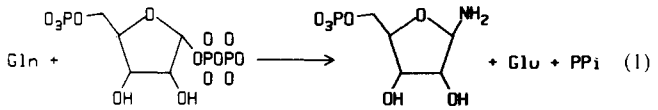
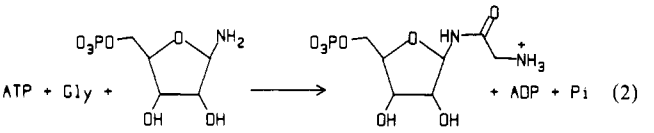
ABSTRACT: Incubation of [1-^{13}C]-5-phosphoribosyl pyrophosphate ([1-^{13}C]PRPP) and glutamine with PRPP amidotransferase results in rapid production and disappearance of two new resonances at 89.3 and 85.9 ppm. These resonances coincide with two of the products produced upon incubation of [1-^{13}C]ribose 5-phosphate with NH_3. Extensive NMR studies (^{15}N and ^{1}H–^{13}C chemical shift correlation spectra) have allowed assignment of these resonances to β- and α-phosphoribosylamine. These studies represent the first spectral observations of this chemically reactive intermediate. The rate of interconversion of α- to β-phosphoribosylamine as a function of pH has been determined by saturation and inversion-transfer NMR methods. The rate of formation of 5-phosphoribosylamine (PRA) from ribose 5-phosphate and NH_3 and its rate of decomposition as a function of pH have been determined with a glycinamide ribonucleotide synthetase trapping system fashioned after earlier studies of Nierlich and Magasanik [Nierlich, D. P., & Magasanik, B. (1965) *J. Biol. Chem. 240*, 366]. Phosphoribosylamine has a $t_{1/2}$ = 38 s at 37 °C and pH 7.5. The pH-independent equilibrium constant for ribose 5-phosphate and NH_3 with phosphoribosylamine has been established, 2.5 M^{-1}, by use of these rate constants as well as by NMR methods. This equilibrium constant and the rates of nonenzymatic interconversion of α- and β-PRA provide essential background for studying the mechanism of glycinamide ribonucleotide synthetase and investigating the possibility of channeling phosphoribosylamine between this enzyme and the first enzyme in the purine pathway.

The first committed step in the purine biosynthetic pathway involves the conversion of 5-phosphoribosyl pyrophosphate (PRPP)[1] and glutamine to 5-phosphoribosylamine (PRA), glutamate, and pyrophosphate by PRPP amidotransferase (eq 1). Although PRA is widely accepted to be the product of



(1)

this amidotransferase, it has never been directly isolated and characterized, presumably due to its chemical instability. The evidence which strongly suggests its existence is based on the trapping of PRA, produced both enzymatically and chemically from the reaction of ribose 5-phosphate (R-5-P) and NH_3, by GAR synthetase. This trapping involves the second enzyme in the purine pathway, GAR synthetase, and its substrates, glycine and ATP (Goldthwait, 1956) (eq 2). This reaction



(2)

produces glycinamide ribonucleotide (GAR), which is stable, is isolable, and has been well characterized (Hartman et al., 1956; Peabody et al., 1956; Chettur & Benkovic, 1977). In addition to the fact that PRA had only been characterized indirectly at the time these studies were undertaken, the "quantitative" stability of PRA was also controversial. In 1956, Goldthwait, measuring NH_3 release as a criterion for PRA decomposition, concluded that between pH 7 and pH 8 only

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[1] Abbreviations: PRPP, 5-phosphoribosyl pyrophosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; R-5-P, ribose 5-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; FID, free induction decay.

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4% of his "putative" PRA decomposed in 30 min at 25 °C. These results are dramatically different from the more thorough and carefully executed experiments of Nierlich and Magasanik (1965), who generated PRA from R-5-P and NH_3 and determined that at 37 °C the $t_{1/2}$ of its decomposition was ≈33 s at pH 7.5. These workers used partially purified GAR synthetase from *Aerobacter aerogenes* as a trap to measure the rate constants of both formation, k_f, and decomposition, k_d, of PRA. However, because of inadequate protocols used in these early investigations, partially due to the lengthy incubation times and insufficient amounts of GAR synthetase, only an underestimate of both the concentration of PRA and hence its rate of formation could be made. The low estimates of PRA concentration are presumably also responsible for establishing only a lower limit on the equilibrium constant for the production of PRA from R-5-P and NH_3.

As a prerequisite for investigating the kinetics of the *Escherichia coli* GAR synthetase[2] and the newly discovered trifunctional protein from chicken liver which contains GAR synthetase (Daubner et al., 1985; Schrimsher et al., 1986), the existence of PRA and its rate of formation, hydrolysis, and anomerization needed to be determined. These tasks have been greatly facilitated because of the recent cloning and overproduction of the enzymes PRPP amidotransferase (Tso et al., 1982) and GAR synthetase.[2] Large quantities of PRPP amidotransferase have facilitated rapid preparation of sufficient quantities of [1-^{13}C]PRA to be characterized spectroscopically for the first time. The cloned and overexpressed GAR synthetase has facilitated trapping of PRA and hence accurate measurement of both its rate of formation and its rate of decomposition. The present paper reports the successful completion of efforts to characterize PRA both by spectroscopic means and in terms of its chemical stability.

Experimental Procedures

Materials. Dowex 50W-X8, Sephadex G-25, DEAE-Sephadex A-25, DEAE-Sepharose CL-6B, QAE-Sephadex A-50, ATP, HEPES, TAPS, CAPS, D-ribose 5-phosphate, D-ribose, L-glutamine, ninhydrin, ampicillin, triethanolamine, 5-phosphoribosyl pyrophosphate, bovine serum albumin, *E. coli* glutaminase (8.4 μmol min^{-1} mg^{-1}), yeast inorganic pyrophosphate (590 μmol min^{-1} mg^{-1}), ammonia standard solution, and ammonia color reagent were obtained from Sigma Chemical Co. Cellex D was purchased from Bio-Rad Laboratories. Deuterium oxide (99.8 atom % ^{2}H), NaO^{2}H (40%, 99+ atom % ^{2}H), ^{2}HCl, (20%, 99+ atom % ^{2}H), and [^{13}C]-methanol (99.7 atom % ^{13}C) were obtained from Aldrich Chemical Co. D-[1-^{13}C]Ribose (99 atom % ^{13}C) was purchased from Omicron Biochemicals, Ithaca, NY. [^{15}N]Ammonium sulfate (99 atom % ^{15}N), [1-^{14}C]glycine (56 mCi/mmol), and D-[1-^{14}C]ribose (58 mCi/mmol) were obtained from Amersham. The [^{32}P]inorganic phosphate used for the synthesis of [γ-^{32}P] ATP was obtained from New England Nuclear Corp. *Klebsiella pneumoniae* ATCC 8724 was obtained from the American Type Culture Collection. *E. coli* strain TX358/pSB5 was a generous gift from Dr. H. Zalkin, Purdue University, West Lafayette, IN. *E. coli* strain HO561/pHO11 (Hove-Jensen, 1985) was a generous gift from Dr. R. L. Switzer, University of Illinois, Urbana, IL. *E. coli* strain TX634/pJS86 containing the gene for GAR synthetase was a generous gift from Dr. J. Smith, Louisiana State University Medical Center, Shreveport, LA. All other reagents were of reagent grade or better unless otherwise specified.

General Methods. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Phosphate was quantitated by the method of Ames and Dublin (1960), and ribose was quantitated by the orcinol assay (Dische, 1962). Ammonia concentrations were determined by the Nessler-based Sigma ammonia assay reagent. Glycine concentrations were determined by a modified ninhydrin assay with L-glutamine as a standard (Rosen, 1957). The concentrations of L-glutamine solutions were determined by quantitation of the ammonia or L-glutamate released by the action of glutaminase (Williamson & Corkey, 1979). Quantitation of PRPP was determined by following the conversion of orotate to uridine 5′-phosphate (Kornberg et al., 1955) [γ-^{32}P]ATP was prepared by the procedure of Selman et al. (1981). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of product per minute at 37 °C. GAR synthetase was assayed by the procedure of Schrimsher et al. (1986). Phosphoriboisomerase was assayed by the procedure of Axelrod and Jang (1954). PRPP synthetase was assayed by the procedure of Switzer and Gibson (1978).

Ribokinase was assayed by following the conversion of [1-^{14}C]ribose to [1-^{14}C]R-5-P. The reaction mixture contained in a final volume of 500 μL 10 mM ATP, 20 mM $MgCl_2$, 40 mM HEPES (pH 7.7), 5 mM [1-^{14}C]ribose (1×10^5 cpm/μmol), and varying amounts of enzyme. The reaction was incubated at 37 °C; 100 μL was withdrawn at 0, 10, 20, and 30 min and applied to a 0.5-mL column of DEAE-Sephadex A-25. The columns were washed with 4 mL of water and then eluted with 3 mL of 250 mM triethylammonium bicarbonate (pH 7.8). The amount of [1-^{14}C]R-5-P produced was then determined by liquid scintillation counting.

PRPP amidotransferase was assayed by coupling the formation of PRA to the production of GAR with GAR synthetase. The reaction mixture contained in a final volume of 200 μL 50 mM Tris-HCl (pH 8.0), 10 mM $Mg(OAc)_2$, 4 mM L-glutamine, 2.5 mM ATP, 2 mM PRPP, 2 mM [1-^{14}C]glycine (2×10^5 cpm/μmol), 0.05 units of GAR synthetase, and varying amounts of PRPP amidotransferase. The reaction was incubated at 37 °C; 40 μL was withdrawn at 0, 2, 4, and 8 min and placed in a microfuge tube containing 15 μL of 30% trichloroacetic acid. The precipitated protein was removed by centrifugation at 6500 *g* for 1 min, and 40 μL of the supernatant was applied to a column (0.5 × 4 cm) of Dowex 50W-X8 (equilibrated in 50 mM ammonium formate, pH 3.3). The column was washed with 2.5 mL of 50 mM ammonium formate (pH 3.3), and the amount of GAR produced was quantitated by liquid scintillation counting.

Instrumentation. Liquid scintillation counting was carried out on a Packard 310 liquid scintillation counter using Packard Scint-A scintillation fluid. Mono-Q anion-exchange chromatography was carried out on a Pharmacia FPLC. Fourier transform NMR spectra were obtained with 5- or 10-mm sample tubes containing 30–100% D_2O as a lock solvent. ^{1}H NMR spectra were obtained on a Bruker WH 270 spectrometer; ^{13}C NMR spectra were obtained at either 50.3 or 62.8 MHz on a Nicolet 200 or a Bruker WM 250 spectrometer, respectively. ^{15}N NMR spectra were obtained at 25.3 MHz on a Bruker WM 250 spectrometer.

NMR Methods. (*A*) *^{13}C NMR.* Solutions of [1-^{13}C]R-5-P were passed through a small column (0.4 × 4 cm) of Dowex 50W-X8 (H^+) and then neutralized with 1 N NaOH and concentrated to dryness in vacuo. Ammonium chloride was repeatedly exchanged with D_2O, and then 0.5 mL of a 2 M

[2] J. E. Smith, Y. S. Cheng, F. J. Schendel, and J. Stubbe, unpublished experiments.

ND_4Cl solution was added to 100 μmol of [1-^{13}C]R-5-P. The pD was determined from eq 3, and adjusted by the addition

$$pD = 0.4 + \text{pH meter reading} \quad (3)$$

of DCl or NaOD. The sample was then filtered into the NMR tube through a glass fiber filter (Whatman GF/A). TAPS buffer (0.5 M) was included in all samples being examined in the pH range 8.0–9.0. Chemical shifts are reported in parts per million downfield from tetramethylsilane and were measured relative to [^{13}C]methanol (49.0 ppm), which was added to each sample as an internal standard. The NMR tube was sealed with a plastic cap and then wrapped with Parafilm. Samples were allowed to equilibrate at room temperature for 30 min before being placed into the NMR probe. After another 20 min of equilibration in the NMR probe, ^{13}C spectra were recorded. All spectra were obtained using bi-level decoupling to minimize sample heating. Temperature measurements were obtained on 2 M ammonium chloride solutions or on actual samples by placing a thermometer into the NMR tube after removal from the probe. These measurements are accurate to within 2 deg. ^{13}C spin-lattice relaxation times (T_1) were measured by the inversion–recovery method (Cutnell et al., 1976).

(*B*) *^{15}N NMR.* A 1.7-mL solution of 1 M R-5-P, 0.5 M TAPS, and 0.6 M [^{15}N]ammonium sulfate containing 15% D_2O was adjusted to pH 9.6 by the addition of 5 N NaOH. The sample was then filtered into a 10-mm NMR tube, capped, sealed with Parafilm, and allowed to equilibrate as described above. Chemical shifts are reported in parts per million downfield from liquid ammonia and were determined relative to nitromethane (380.2 ppm), which was used as an external standard.

Equilibrium Measurements. The samples were prepared as described above and typically contained 200–400 mM [1-^{13}C]R-5-P, 2 M ammonium chloride, and 500 mM buffer. The NMR spectra were acquired as described below, and after data collection on each sample was completed, 1 μL was removed and added to 1 mL of 400 mM sodium acetate (pH 4.0) for determination of ammonia and R-5-P concentrations. The pH of the sample was also checked to determine if any change had occurred during the experiment. At least five spectra were obtained for each measurement, and the equilibrium amounts of each species were determined by cutting and weighing each peak. All spectra were obtained at 50.3 MHz with 8K data points, 4000-Hz spectral width, 65° pulse angle, and continuous broad-band proton decoupling. An average of 800 acquisitions was accumulated for each spectrum. Exponential multiplication with a line-broadening factor of 2 Hz was applied to each FID before Fourier transformation. The validity of this rapid pulse method was checked by comparing the results with those obtained by the more time consuming 90° pulse–$5T_1$ delay method to generate "fully relaxed" spectra and with gated decoupled spectra in which the nuclear Overhauser enhancement was eliminated. The relative intensities of the resonances were not significantly different with any of these methods, and therefore, the rapid pulse technique was used throughout the pH and temperature range examined.

Saturation-Transfer Measurements. Saturation-transfer experiments were performed at 62.8 MHz (^{13}C) with steady-state magnetization transfer as described by Alger and Schulman (1984). The saturating pulses of 3-s duration were applied during the delay between the observed pulses (90° pulse angle, 1-s acquisition time). The saturating frequency was generated by a PTS-160 frequency synthesizer and amplified and gated by a Bruker BSV-3 decoupler unit. To determine whether the saturating frequency was affecting other resonances in the spectrum, control spectra were obtained with the position of the off-resonance saturating frequency offset by an equivalent amount to the upfield and downfield side of the resonance of interest. Difference spectra between these controls and the on-resonance spectra were identical. When the resonances of species B is saturated, the rate constant k_1 for the reaction

$$\underset{(\alpha\text{-PRA})}{\text{A}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(\beta\text{-PRA})}{\text{B}} \quad (4)$$

was calculated according to

$$k_1 = (\Delta M_A / M_A^0) R_{obsd} \quad (5)$$

where ΔM_A represents the change in magnetization of A during saturation of B, M_A^0 represents the initial magnetization of A in the absence of saturation of B, and R_{obsd} represents the inverse of the time constant for inversion recovery of A during saturation of B (Alger & Shulman, 1984). $R_{obsd} = R_1 + k_1$, where $R_1 = 1/T_1$ in the absence of exchange.

Inversion-Transfer Measurements. Inversion-transfer experiments were performed at 50.3 MHz (^{13}C), and the α resonance of PRA was selectively inverted with the pulse sequence $\pi/2-\tau_1-\pi/2-\tau_2-\pi/2$ described by Robinson et al. (1985). At least 15 delay times were used for each sample at each temperature and pH. The data were fit by nonlinear least-squares regression to

$$M_B(t) = M_B^E + M_B^0\phi_1 \exp(-\lambda_1 t) - M_B^0\phi_2 \exp(-\lambda_2 t) \quad (6)$$

where M_B is the z component of the nuclear magnetization of species B, M_B^E represents the unperturbed equilibrium magnetization of B, and λ_1, λ_2, ϕ_1, and ϕ_2 are constants defined previously (Robinson et al., 1984). From this analysis, values for λ_1, λ_2, ϕ_2, and ϕ_1 are obtained. The rate constant is then obtained from

$$k_1 = (\lambda_1 + \lambda_2 - 1/T_1^A - 1/T_1^B)/(1 + K_{eq}) \quad (7)$$

where T_1^A and T_1^B are the spin–lattice relaxation times measured in the absence of chemical exchange between A and B and $K_{eq} = k_1/k_{-1}$ (Robinson et al., 1985).

Isolation of Ribokinase. Klebsiella pneumoniae (ATCC 8724) was cultured on media described by Horecker (1957) modified to contain 6 g/L K_2HPO_4, 2 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 1 g/L NaCl, and 0.4 g/L $MgSO_4 \cdot 7H_2O$ adjusted to pH 7.4 and supplemented with 2 g/L D-ribose and 2 g/L Difco casamino acids. The cells were grown at 37 °C with agitation and harvested in late log phase, yielding 1–2 g of cells per liter of media. The cells were frozen with liquid nitrogen and stored at −80 °C.

Cells (4 g) were resuspended in 5 volumes (20 mL) of 50 mM Tris-HCl, pH 7.5, and passed through a French press pressure cell at 16 000 psi. The suspension obtained was centrifuged (18000*g*) for 20 min. The supernatant was filtered through cheesecloth, and ammonium sulfate (0.277 g/mL, 45% saturation) was added over 10 min. The solution was stirred for an additional 30 min and then centrifuged (18000*g*) for 20 min. The supernatant was separated and subjected to gel filtration on a column of Sephadex G-25 (4 × 40 cm) equilibrated with 50 mM Tris-HCl, pH 7.5.

The desalted protein was applied to a column of Cellex-D (2.5 × 9 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.5. The column was then washed with the sample buffer until the absorbance at 280 nm of the effluent was less than 0.2. The enzyme was then eluted with a 1-L linear gradient of KCl from 0 to 400 mM in 50 mM Tris-HCl, pH 7.5. Fractions containing ribokinase activity eluted at 150 mM

KCl, were pooled, and were concentrated to a volume of 15 mL on an Amicon ultrafiltration apparatus with a PM-30 membrane.

The concentrated protein was then applied to a column of QAE-Sephadex (2.5 × 4 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.5, and 120 mM KCl. The column was washed with the sample buffer until the absorbance at 280 nm of the effluent was less than 0.1 and then eluted with 50 mM Tris-HCl, pH 7.5, containing 160 mM KCl. Fractions that contained ribokinase activity were pooled, dialyzed against 50 mM Tris-HCl buffer, pH 7.5, and then concentrated as described above. At this stage, the protein was pure enough to use in the biosynthesis of R-5-P (0.35 unit/mg) (no phosphoriboisomerase activity was observed).

However, enzyme of 5-fold higher specific activity could be obtained by use of an FPLC Mono-Q anion-exchange column. The column was equilibrated with 50 mM Tris-HCl (pH 7.5) containing 100 mM KCl and developed with a (50-mL) linear gradient from 100 to 300 mM KCl in 50 mM Tris-HCl buffer (pH 7.5). The ribokinase activity which eluted at 250 mM in the gradient was pooled and concentrated as described above (1.6 units/mg). the enzyme was stored at −20 °C in 50 mM Tris-HCl (pH 7.5) containing 25% glycerol.

Isolation of PRPP Synthetase. *E. coli* strain HO561/pH011 was grown on media containing 5 g/L Bacto yeast extract, 10 g/L Bacto tryptone, and 10 g/L NaCl adjusted to pH 7.4 supplemented with 2 g/L glucose and 50 μg/mL ampicillin. The cells were grown at 37 °C with shaking and harvested in late log phase to yield approximately 3 g of cells per liter of media. PRPP synthetase was purified through the first ammonium sulfate precipitation step by the procedure of Switzer and Gibson (1978). This procedure gave an 80% recovery of PRPP synthetase activity with a specific activity of 26 μmol min^{-1} mg^{-1}.

Isolation of GAR Synthetase. The cloning, overexpression, isolation, and characterization of GAR synthetase from *E. coli* will be described elsewhere. The specific activity of GAR synthetase is 18 μmol min^{-1} mg^{-1} (J. E. Smith, Y. Cheng, F. Schendel, and J. Stubbe, unpublished experiments).

Isolation of PRPP Amidotransferase. *E. coli* strain TX358/pSB5 was grown and PRPP amidotransferase was isolated as previously described for *E. coli* strain TX158/pSB2 (Tso et al., 1982; Messenger & Zalkin, 1979). The specific activity of PRPP amidotransferase was 25 μmol min^{-1} mg^{-1}.

Preparation of [1-^{13}C]R-5-P. A typical reaction mixture (36 mL) containing 40 mM Tris-HCl (pH 7.5), 6 mM D-[1-^{13}C]ribose, 10 μCi of D-[^{14}C]ribose, 10 mM ATP, 12 mM $MgSO_4$, and 16.4 units of ribokinase was incubated at 37 °C for 30 min. The reaction was quenched by the addition of 4 mL of 30% trichloroacetic acid, and the precipitated protein was removed by centrifugation at 6000*g*. The pH of the supernatant was readjusted with 5 N KOH to 7.8 and loaded onto a DEAE-Sephadex A-25 column (2.5 × 20 cm). The column was eluted with a 1-L linear gradient of (0–400 mM) triethylammonium bicarbonate (pH 7.8). Fractions which contained radioactivity were pooled and evaporated to dryness in vacuo. The product was then converted to its sodium salt by dissolving it in 5 mL of water and passing it through a small column (5 mL) of Dowex 50W-X8 (Na^+) resin. The combined effluent and washings (40 mL) were concentrated to dryness in vacuo to give 197 μmol of D-[1-^{13}C]R-5-P (90%): ^{1}H NMR (D_2O; HOD, δ 4.40) δ 4.82 (1 H, d, J_{CH} = 174 Hz, 1-β-H), 5.00 (1 H, d, J_{CH} = 174 Hz, 1-α-H) (the 2-, 3-, 4-, and 5-hydrogens of the sugar appear between δ 3.28 and δ 3.88); ^{13}C NMR {^{1}H} (D_2O; using dioxane as standard, δ 66.50) δ 96.09 (1-α), 101.07 (1-β).

Synthesis of [1-^{13}C]PRPP. The reaction mixture (15 mL) containing 200 μmol of $MgCl_2$, 120 μmol of ATP, 120 μmol of [1-^{13}C]R-5-P, 2.5 μCi of [1-^{14}C]R-5-P, 140 units of PRPP synthetase, 0.033 M triethanolamine, 0.033 M potassium phosphate (pH 7.5), and 0.67 mM EDTA, was incubated at 37 °C for 10 min. The pH was then lowered to 5.6 by the addition of 1 N acetic acid. The reaction was cooled to 4 °C, 500 μL of 20% acid washed charcoal suspension added, and the mixture placed on ice. After 2 min, the charcoal was separated by centrifugation at 5000*g*. The pH of the supernatant was readjusted to 7.5 and then applied to a column of DEAE-Sephadex A-25 (2.5 × 18 cm). The column was developed with a 1.1-L linear gradient of (0–700 mM) triethylammonium bicarbonate (pH 7.8). The fractions which contained PRPP eluted at 600 mM in the gradient and were located by scintillation counting, pooled, and concentrated to dryness in vacuo. The amount of PRPP was then quantitated as described above: yield 109 μmol (90%); ^{1}H NMR (HOD, δ 4.4) δ 5.50 (ddd, 1 H, J_{CH} = 179 Hz, $^3J_{PH}$ = 5 Hz, J_{1-2} = 5 Hz); ^{13}C NMR {^{1}H} [D_2O, pD 8.5, 0.1; (v/v) $^{13}CH_3OH$, δ 49.0] δ 98.4 (d, $^2J_{CP}$ = 5.2 Hz).

Conversion of [1-^{13}C]PRPP to [1-^{13}C]PRA. Into a 5-mm NMR tube was placed 40 μmol of [1-^{13}C]PRPP, 80 μmol of L-glutamine, 80 μmol of $MgCl_2$, 1 μL of [^{13}C]methanol, 1000 units of inorganic pyrophosphatase, 0.4 μmol of EDTA, and 400 μL of 250 mM TAPS (pH 8.5) (50% D_2O). The tube was placed into the NMR probe and equilibrated to 15 °C, and a ^{13}C NMR spectrum was recorded (Figure 3A). The tube was removed from the probe, and 200 units of PRPP amidotransferase in 100 μL of 10 mM Tris-HCl (pH 8.0) was added, the tube was placed back into the probe, and the spectra were accumulated every 2 min (Figure 3B,C). When all of the PRPP had been converted to R-5-P, the accumulations were stopped, and the pH and temperature of the sample were measured.

Rate of Formation of PRA. A typical reaction mixture contained in a final volume of 0.65 mL 50 mM TAPS/CAPS from pH 8.0 to pH 9.5 or 50 mM HEPES from pH 7.0 to pH 8.0, 20 mM [1-^{14}C]glycine (3.7 × 10^5 cpm/μmol), 6 mM $Mg(OAc)_2$, 3.4 mM ATP, 40 mM NH_4Cl, 25 mM R-5-P, and 1.1 units of GAR synthetase. At pH 9.50 only 20 mM NH_4Cl was used. Due to the lower activity of GAR synthetase at higher pH, 2.2 units of GAR synthetase was used above pH 9.0. All these reactants except R-5-P were preincubated for 5 min, and the reaction was initiated by the addition of R-5-P. At varying time intervals, 100 μL of the reaction mixture was withdrawn and quenched with 15 μL of 30% (w/v) trichloroacetic acid. The amount of GAR produced was quantitated by the method of Schrimsher et al. (1986). The concentration of NH_3 was calculated from pK_a = 9.25 and the measured pH. The rate constant for formation, k_f, of PRA was calculated by dividing the measured initial rate of production of GAR by the appropriate concentrations of R-5-P and NH_3.

Rate of Hydrolysis of PRA. PRA was formed by incubation of 125 mM R-5-P and 125 mM NH_4Cl at pH 10 at 24 °C for 45 min. Then, 50 μL of the equilibrium mixture containing PRA was withdrawn and added to a sealed vial equipped with a magnetic stirrer containing 450 μL of buffer [50 mM TAPS/CAPS (pH 8–9) or 50 mM HEPES (pH 7–8)]. At different time intervals, 30 μL of the reaction mixture was withdrawn and added into an assay solution (120 μL) which contained 100 mM Tris-HCl (pH 8.0), 6 mM $Mg(OAc)_2$, 5 mM ATP, 2 mM [1-^{14}C]glycine (4.60 × 10^6

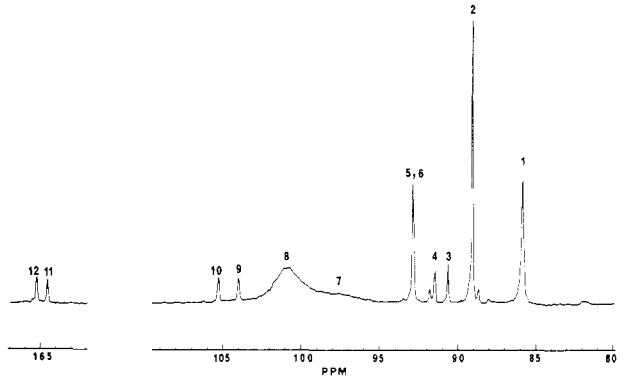


FIGURE 1: Proton-decoupled 62.8-MHz ^{13}C NMR spectrum showing the products formed upon reaction of 400 mM [1-^{13}C]R-5-P and 2.0 M NH_4Cl at pH 9.3 and 20 °C.

cpm/μmol), and GAR synthetase (0.27 unit). This mixture was incubated at 24 °C for 5–15 s and then quenched with 15 μL of 30% trichloroacetic acid, and the GAR was quantitated as described above. The rate constant for decomposition of PRA, k_d, was determined by plotting log of the percent remaining PRA vs time. The K_{eq} could be calculated from k_f/k_d.

Equilibrium Constant for PRA Formation. An equilibrium mixture of R-5-P, ammonia, and PRA was formed by incubating 10 mM R-5-P and 250 mM NH_4Cl in 100 mM TAPS buffer at pH 8.3 for 45 min. To determine the amount of R-5-P at equilibrium, 4 μL of this mixture was withdrawn and added to a reaction mixture (246 μL), which had been preincubated at 37 °C for 5 min, containing 10 mM $MgCl_2$, 5 mM ATP, 50 mM potassium phosphate, 50 mM triethanolamine, 1 mM EDTA, and 0.35–0.7 unit of PRPP synthetase at a final pH of 7.5. After 5 s, the reaction was quenched by the addition of ice cold 5% $HClO_4$, 80 μL, and then immediately reneutralized by the addition of ice cold 5% KOH, 80 μL. The amount of PRPP formed was determined as described above after removal of the $KClO_4$ by centrifugation. Control experiments were performed to show that this quenching procedure did not destroy any PRPP. The amount of PRA in the equilibrium mixture was determined as described for the rate of hydrolysis experiments, and the amount of ammonia was determined by the Nessler-based assay. The equilibrium constant was then calculated from the expression $K_{eq} = [PRA]/[R\text{-}5\text{-}P][NH_3]$.

Results and Discussion

Spectroscopic Identification of PRA. In order to investigate the chemical stability of PRA, it was necessary to have a reliable method to prepare this molecule. Early work by Nierlich and Magasanik (1965) had suggested that incubation of R-5-P and NH_4Cl or NH_4OH in aqueous solution resulted in the formation of PRA. Our first attempt at preparation of PRA for analysis by ^{13}C NMR spectroscopy therefore utilized this approach. In order to simplify interpretation of the spectrum, [1-^{13}C]R-5-P was prepared by utilization of ribokinase from *Klebsiella pneumoniae*, ATP, and [1-^{13}C]-ribose.[3] Incubation of [1-^{13}C]R-5-P and NH_4Cl at pH 9.3 resulted in the spectrum shown in Figure 1. The broad resonances at 100.8 and 97.5 ppm are assigned to the β- and α-anomers of R-5-P, respectively. These resonances became increasingly broaded as the pH is raised above 8.0 owing to an increase in their rate of interconversion (Pierce et al., 1985).

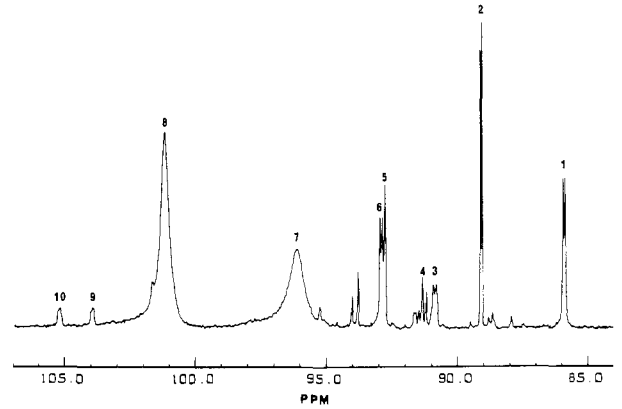


FIGURE 2: Proton-decoupled 62.8-MHz ^{13}C NMR spectrum showing the products formed from reaction of [1-^{13}C]R-5-P and [^{15}N]ammonium sulfate at pH 8.6 and 20 °C.

Table I: NMR Data for the Reaction of R-5-P with Ammonia

resonance[a]	^{13}C shift (ppm)[a]	^{1}H shift (ppm)[b]	$^{1}J_{CN}$ (Hz)[c]	^{13}C T_1 (s)[d]
1	85.9	4.94	4.8	0.42
2	89.3	4.65	5.0	0.44
3	90.8	5.55	8.0	0.16
4	91.5	5.05	8.0	0.15
5	92.8	4.93		0.17
6	92.9	4.62	5.4	0.18
7	97.5	5.40		0.47
8	100.8	5.25		0.46
9	104.1	ND[e]	4.8	0.18
10	105.4	ND	5.0	0.18
11	164.3	ND	ND	ND
12	165.2	ND	ND	ND

[a] The ^{13}C chemical shifts and numbering scheme are from Figure 1. [b] The chemical shifts of the anomeric protons attached to labeled C-1 carbons were measured indirectly from a ^{1}H–^{13}C chemical shift correlation spectrum. [c] One-bond ^{13}C–^{15}N couplings were obtained from the ^{13}C spectrum in Figure 2. [d] T_1 values were measured at 62.8 MHz at 24 °C at pD 8.84. [e] ND, not determined.

In addition, major resonances attributable to labeled C-1 carbons were observed at 85.9, 89.3, 90.8, 91.5, 92.8, 104.1, 105.4, 164.3, and 165.2 ppm and are assigned as discussed below.[4]

The first step in the assignment process was to determine which resonances arise from C-1 carbons bonded to nitrogen instead of oxygen. This was established by incubating [1-^{13}C]R-5-P with [^{15}N]ammonium sulfate at pH 8.6 and determining which resonances now exhibited ^{13}C–^{15}N scalar coupling. As shown in Figure 2, all of the carbon resonances except numbers 5, 7, and 8 are coupled to ^{15}N. The coupling constants are listed in Table I.

Resonances 1 and 2 at 85.9 and 89.3 ppm are assigned to the C-1 of α- and β-PRA, respectively, on the basis of several lines of evidence. First, as expected for replacement of O with N at C-1, these resonances are found upfield from the R-5-P resonances and are coupled to ^{15}N ($^{1}J_{CN}$ = 4.8 Hz) as would be expected for the proposed structures (Wasylishen, 1977). More direct evidence was provided in an experiment in which PRA was enzymatically synthesized from [1-^{13}C]PRPP and glutamine with PRPP amidotransferase. This approach, although technically difficult because of the reported instability of PRA (Nierlich & Magasanik, 1965), was thought to be feasible because of the recent cloning and overproduction of PRPP amidotransferase from *E. coli* by Tso et al. (1982). The

[3] Recent cloning and overproduction of ribokinase from *E. coli* (Hope et al., 1986) has now greatly facilitated this approach.

[4] Control experiments under identical conditions in the absence of ammonia showed only resonances attributable to R-5-P.

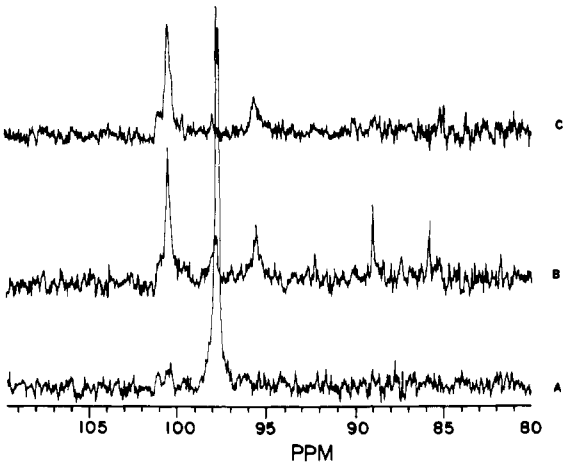


FIGURE 3: Proton-decoupled 50.3-MHz ^{13}C NMR spectra showing the conversion of [1-^{13}C]PRPP to the 86 and 89 ppm resonances by PRPP amidotransferase and the decomposition of these resonances to R-5-P. [1-^{13}C]PRPP before the addition of PRPP amidotransferase (A) and 4 min (B) and 10 min (C) after addition of PRPP amidotransferase.

results of a typical experiment are shown in Figure 3. The large doublet at 97.7 ppm, due to [1-^{13}C]PRPP (Figure 3A), is shown to decrease with time (Figure 3B,C). Two resonances at 85.9 and 89.3 ppm appear (Figure 3B) and disappear (Figure 3C) rapidly as a function of time, as would be expected for α- and β-PRA. The resonances which appear and remain at 95.4 and 100.5 ppm are assigned to α- and β-R-5-P, which are produced upon rapid hydrolysis of PRA. Although the β-anomer of PRA is the proposed product of the reaction catalyzed by PRPP amidotransferase (Goldthwait et al., 1955), the anomerization rate of PRA, as will subsequently be shown, is more rapid than the hydrolysis rate. Therefore, both the α and β resonances of PRA are observed.

The assignment of resonance 1 to α-PRA and resonance 2 to β-PRA is based on several considerations. First, the extensive literature of ^{13}C chemical shifts of furanosides and cyclopentanols indicates that the cis configuration of vicinal substituents gives resonances that are generally more shielded than the corresponding trans configuration (Ritchie et al., 1975). In addition, the trans arrangement of the 1- and 2-substituents of β-PRA is expected to be more stable, which should result in a greater equilibrium concentration of the β-anomer. Therefore, the observed 60:40 ratio for resonances 2 and 1 is consistent with both the proposed stability and the chemical shifts for the β- and α-anomers of PRA. Additional evidence in support of the α,β-anomeric assignments was obtained from a two-dimensional ^1H–^{13}C chemical shift correlation spectrum of R-5-P, NH_3, and PRA at pH 8.6. The chemical shift of the proton attached to the carbon giving rise to resonance 2 was found to be 4.65 ppm, while the proton bonded to carbon 1 comes at 4.95 ppm (Table I). The anomeric proton resonance for β-PRA is predicted to be upfield from that of α-PRA due to the shielding effect of the 2′-hydroxyl group (Nishimura et al., 1962). In addition, recent studies from our laboratory in which a variety of glycinamide ribonucelotide analogues have been prepared indicate that the resonance for the 1-H of the α-anomer is always downfield from the 1-H of the β-anomer (Schendel & Stubbe, 1986). Thus, both the ^{13}C and ^1H chemical shift data are consistent with the proposed assignments. These assignments are also consistent with ^{13}C NMR studies of Kam and Oppenheimer (1979) on 5-*O*-trityl-D-*aldo*-pentofuranosylamines in CD_3OD.

Other Products from Reaction of R-5-P and NH_3. The additional resonances besides those assigned to R-5-P and PRA

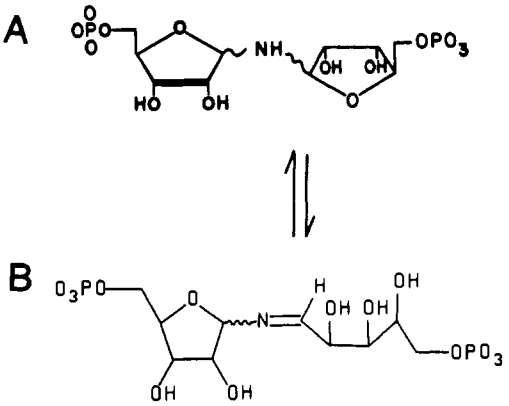


FIGURE 4: Proposed structures for the dimer species giving rise to the additional resonances in the ^{13}C and ^{15}N NMR spectra of R-5-P and NH_3: (A) PRA dimer; (B) ring-opened imine form of the PRA dimer.

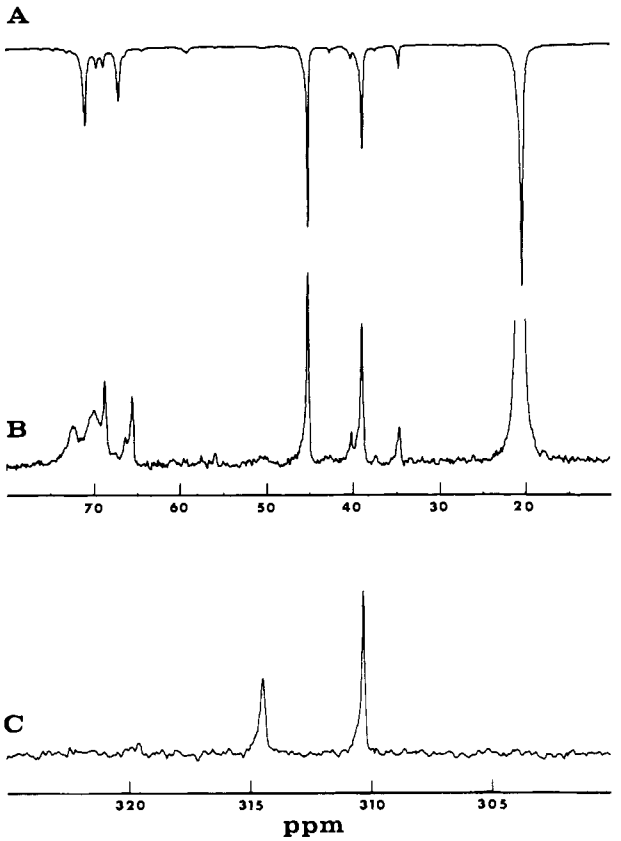


FIGURE 5: ^{15}N NMR spectra at 25.3 MHz of 1 M R-5-P and 0.6 M [^{15}N]ammonium sulfate in 0.5 M TAPS buffer, pH 9.6. (A) Amine region of proton-decoupled spectrum at 5 °C. Resonances are inverted due to the negative nuclear Overhauser enhancement. (B) Amine region of proton-coupled spectrum at 5 °C. (C) Imine region of a proton-decoupled spectrum acquired with gated decoupling to eliminate the negative nuclear Overhauser enhancement.

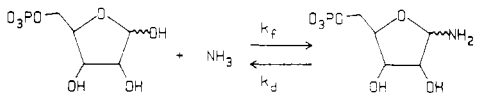
in Figures 1 and 2 indicate that there are several other products formed upon reaction of R-5-P and NH_3. Although it is beyond the scope of this paper to assign each of these resonances, there is literature precedent (Tipson, 1961) and supporting information from ^{13}C T_1 relaxation data, ^{15}N NMR, and chemical degradation studies that most, if not all, of these other products are dimeric species (Figure 4) resulting from the reaction of two molecules of PRA or from reaction of R-5-P with PRA. The involvement of PRA as a precursor to these products is suggested by the observation that the unassigned resonances in the spectrum always appear subsequent to the production of PRA.

Evidence for dimeric products was provided by ^{15}N NMR spectra (Figure 5). In addition to resonances from ammonia (20.2 ppm), α-PRA (39.0 ppm), and β-PRA (45.3 ppm) in the proton-decoupled spectrum in Figure 5A, there are two major and two minor resonances which appear farther downfield between 67 and 71 ppm. In the low-temperature proton-coupled spectrum in Figure 5B, each of these resonances is split into a doublet by one-bond ^{15}N–^{1}H coupling, confirming that they arise from secondary amine nitrogens of the type expected for PRA dimers. It is interesting to note that similar proton coupling to the primary amine nitrogens of PRA is not observed owing to more rapid proton exchange.

Two resonances attributable to imine species are observed in both the ^{13}C spectrum at 164.3 and 165.2 ppm (Figure 1) and the ^{15}N spectrum at 310.2 and 314.7 ppm (Figure 5C). It is important to know whether these imines correspond to the ring-open form of PRA or a ring-open form of a dimeric species (Figure 4). ^{13}C and ^{15}N saturation-transfer experiments conducted under the same conditions which showed exchange between α- and β-PRA (see subsequent section) failed to reveal any transfer of magnetization between PRA and the imine resonances. These results suggest that the observed imines are not intermediates in the interconversion of the two anomers of PRA. Rather, we postulate that they are dimeric species having structures similar to that shown in Figure 4. Such structures might also be the origin of C-1 resonances 9 and 10 at 104.1 and 105.4 ppm, since an imine nitrogen would be expected to induce a relatively downfield shift of the anomeric carbon.

Chemical degradation studies have provided further support for dimer production. Incubation of [^{14}C]R-5-P (206 mM) and NH_4Cl (2 M) at pH 8.9 resulted in isolation from an anion-exchange column of 14% of the material in the diphosphate region. Assay for ribose, phosphate, and NH_3 at pH 7.8 gave a ratio of respective products of 1:1:0. However, assay at pH 5.0 gave a ratio of products of 1:1:0.5. These results are consistent with a dimeric structure(s) and its (their) expected stability(ies). An additional experiment was carried out to show that these unidentified resonances are in slow exchange (with respect to the NMR time scale) with R-5-P. If a solution with species similar to those observed in Figure 1 is acidified to pH 5.0, all of the resonances are very rapidly converted to *only* R-5-P. In fact, R-5-P is recovered in greater than 90% yield at the end of each NMR experiment. This result (observed reversibility) and the lack of observed ^{13}C resonances between 37 and 47 ppm indicate that the undefined resonances (90–100 ppm) are not the result of an Amadori rearrangement (Isbell & Frush, 1958). Thus, a variety of methods indicate that when R-5-P and NH_3 are allowed to equilibrate in solution, many products are formed, some of which are probably dimers of PRA.

Equilibrium Measurements. In order to study the kinetics of the GAR synthetase catalyzed reaction (eq 2), a method is needed to generate known concentrations of PRA. One method is to use R-5-P and NH_3 to produce PRA as originally proposed by Nierlich and Magasanik (1965) (eq 8). Knowing



(8)

the concentration of NH_3 and R-5-P and the K_{eq} allows calculation of the concentration of PRA. An attempt was therefore made, with NMR spectroscopic methods, to measure the pH-independent equilibrium constant for this reaction (eq 8) by comparing the ratio of α- plus β-PRA resonances to those

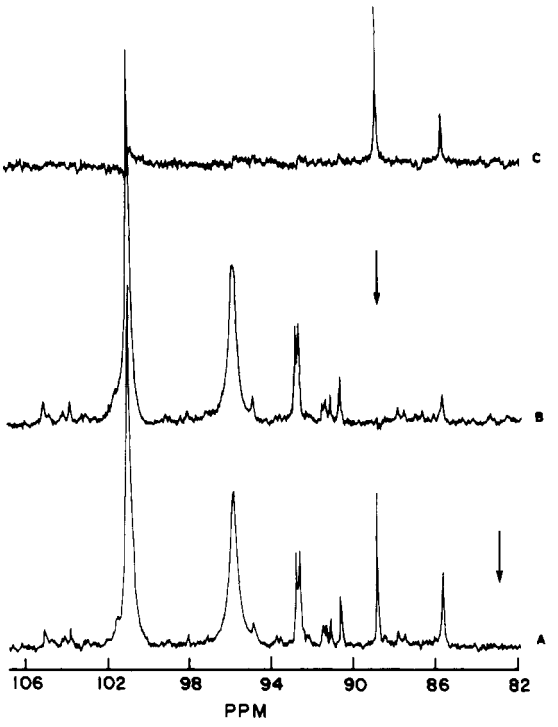


FIGURE 6: Proton-decoupled 62.8-MHz ^{13}C NMR saturation-transfer experiment with the irradiation frequencies indicated by the arrows. (A) Control spectrum; (B) irradiation of the β-PRA resonance; (C) the difference spectrum, generated by subtraction of (A) from (B). The spectra were obtained at 15 °C with 250 mM [1-^{13}C]R-5-P and 1.9 M ND_4Cl at pD 9.1.

of α- plus β-R-5-P resonances. Nine different experiments were carried out in the pH range of 8.0–9.7. At least five spectra were taken at each pH value. The calculated equilibrium constant from the ^{13}C NMR data at 24 °C was 0.95 ± 0.14 M^{-1}. The value is larger than the lower limit of 0.7 M^{-1} placed on the equilibrium constant by early studies of Nierlich and Magasanik (1965) and is substantially lower than the value of 2.5 M^{-1} calculated from the measured rate constants for its formation and decomposition described below. The discrepancy between our observed and calculated values will be discussed subsequently.

Anomerization of PRA. Magnetization-transfer techniques in NMR spectroscopy allow measurement of the rate of exchange at equilibrium between chemically distinct sites. The technique is applicable to systems in which the exchange on the NMR time scale is intermediate to slow. We have utilized both steady-state and temporal magnetization-transfer experiments to measure the rate of interconversion of α- to β-PRA. From the preceding discussion and as indicated in Figure 1, it is clear that α- and β-R-5-P and α- and β-PRA exist in equilibrium with each other and with a complex mixture of partially characterized products. A control experiment, therefore, was run to determine whether on the NMR time scale α- and β-PRA are in exchange with these numerous other species or whether they can be treated as a simple two-site exchange system. An equilibrium mixture of R-5-P, NH_3, and PRA at pD 9.1 was irradiated at 83 ppm, 3 ppm upfield from the α-PRA resonance, and the spectrum was recorded (Figure 6A). The sample was then irradiated at 89.3 ppm (β-PRA resonance), and the spectrum was again recorded (Figure 6B). The difference spectrum (Figure 6C), generated by subtracting the control spectrum (Figure 6A) from that in Figure 6B, shows that only the α-PRA resonance was affected. This result and similar experiments performed at alternate pHs and temperatures indicate that we are dealing

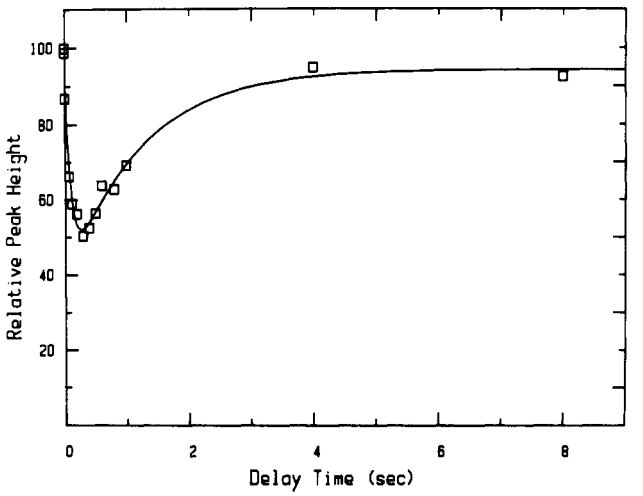


FIGURE 7: Plot of β-PRA peak height, obtained from an inversion-transfer experiment, vs delay time at pD 8.63 and 18 °C (□). The solid line was generated by nonlinear least-squares regression analysis of eq 6 with the data.

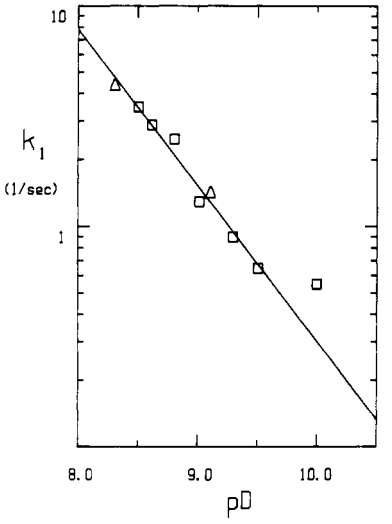


FIGURE 8: Plot of α- to β-PRA anomerization rate at 15 °C vs pD obtained from inversion-transfer (□) or saturation-transfer (Δ) experiments.

with a simple two-site exchange problem, and therefore, interpretation of the saturation-transfer and inversion-transfer experiments is straightforward.

In addition to saturation transfer, the rate of conversion of α-PRA to β-PRA was also investigated with inversion transfer. The inversion-transfer experiments were carried out with selective inversion of the α-PRA resonance over the temperature range of 283–294 K and in the pH range 8.2–9.5. Figure 7 shows the results of a typical experiment performed at 291 K and pD 8.6 in which the relative peak height of β-PRA is plotted as a function of delay time. The solid line was generated by fitting the data with nonlinear least-squares regression analysis to eq 6. From the determined λ_1, λ_2, T_1's, and the K_{eq} the rate of interconversion of the α-anomer of PRA to the β-anomer was calculated with eq 7. The pD dependence of the anomerization rates is shown in Figure 8. Also included in Figure 8 are the results from several saturation-transfer experiments. The results using both protocols are in good agreement and indicate that the rate constant, k_1, for the conversion of α- to β-PRA is 3.6 s^{-1} at pD 8.5 (15 °C). The rate of this conversion increases the decreasing pH and increasing temperature. An Arrhenius plot (data not shown) gave E_a = 23 kcal/mol and a value for $A = 1.1 \times 10^{18}$. Unfortunately, the rates of anomerization in the pH range 7–8

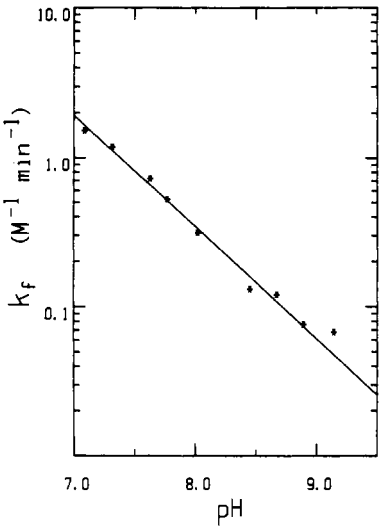


FIGURE 9: Semilog plot of the second-order rate constant for PRA formation at 24 °C vs pH.

are too rapid to be measured by this technique. A mechanism to account for the observed rates of anomerization and hydrolysis of PRA as a function of pH will be proposed and discussed below.

Rate of Formation of PRA. As indicated earlier, in order to investigate the kinetics of GAR synthetase, known amounts of PRA must be accessible. Given the complexity of the products in the equilibrium mixture of R-5-P and NH_3 and the errors involved in determination of peak areas by weight and the corresponding errors in the K_{eq}, an alternative method to determine K_{eq} was sought. The approach taken was to use GAR synthetase as a trap to measure both the rate of formation and the rate of decomposition of PRA. These numbers would then allow calculations of K_{eq}. This method is analogous to the one used by Nierlich and Magasanik in 1965. However, the availability of large amounts of pure GAR synthetase alleviated many of the problems encountered with the procedures used in these early studies.

The protocol used to measure k_f for PRA is analogous to any coupled enzymatic assay. Buffer, Mg^{2+}, ATP, NH_4Cl, [^{14}C]glycine, and GAR synthetase were equilibrated at 24 °C, and the reaction was initiated by addition of R-5-P. The rate of GAR production was shown to be independent of the amount of GAR synthetase present. Periodically, aliquots were removed from the incubation mixture, and the amount of GAR produced was determined by the method of Schrimsher et al. (1986). The formation of GAR was shown to be first order in NH_3, over the range 0.7–78 mM, and first order in R-5-P, over the range 0.43–6 mM. The rate of formation of GAR = $k_f[NH_3][R\text{-}5\text{-}P]$. The results of these studies are summarized in Figure 9. At pH 7.0, the second-order rate constant for the formation of PRA at 24 °C is 1.93 M^{-1} min^{-1}. Furthermore, it is clear from these data that the rate of formation decreases with increasing pH.

Rate of Decomposition of PRA. The protocol was again patterned after the one described by Nierlich and Magasanik, but extensively modified, and allows measurement of the rate of disappearance of PRA. R-5-P and NH_3 are incubated at pH 10 to produce PRA. This PRA-containing solution is then diluted 10-fold into the appropriate buffer. Periodically, aliquots are removed from this solution and added to a trapping mixture containing GAR synthetase, which is then incubated for 5–15 s to convert all of the PRA to GAR. The amount of GAR produced is then determined by the procedure of Schrimsher et al. (1986). The results indicate that the reaction

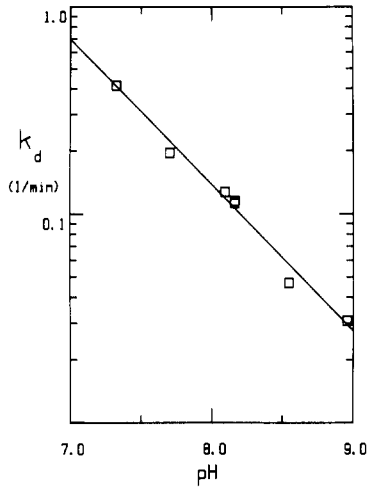


FIGURE 10: Semilog plot showing the rate constant for PRA decomposition at 24 °C as function of pH.

is first order for at least two half-lives. A second experiment in which PRA was generated enzymatically with PRPP, glutamine, and PRPP amidotransferase gave identical results with those in which PRA is generated chemically. In addition, several control experiments in which the number of units of GAR synthetase and the incubation time of the GAR synthetase trap were varied gave identical rate constants. This general scheme, therefore, appears to reliably measure the rate of loss of PRA.

The results of a series of similar experiments in which the pH was varied (Figure 10) indicate that, as in the case of anomerization and formation, the rate of decomposition increases with decreasing pH. The rate constant for the decomposition of PRA at 24 °C and pH 7.5 is 0.31 min^{-1}; that is, PRA has a half-life of 2.2 min. The half-life is reduced to 38 s at 37 °C, as determined from a plot of log of the rate constant of decomposition of PRA as a function of temperature (data not shown). This number is comparable to the half-life of PRA reported by Nierlich and Magasanik at pH 7.5 and 37 °C to be 33 s.

In the preceding sections, the rates of formation and decomposition of PRA under defined conditions were measured. These rate constants (k_f, k_d) have now allowed us to calculate a pH-independent equilibrium constant of 2.5 M^{-1} for the reaction of R-5-P and NH_3 to form PRA. When the equilibrium constant was determined independently by measuring the equilibrium concentrations of R-5-P, NH_3, and PRA at pH 8.35 (described under Experimental Procedures), a value of 2.7 M^{-1} was obtained. This value is in good agreement with the value calculated from the k_f and k_d for PRA. These values, however, differ from the value of 0.95 M^{-1} determined by ^{13}C NMR spectroscopy. Low sensitivity of the NMR method requires the use of very high, nonphysiological concentrations of NH_3 and R-5-P (typically 1 M and 300 mM, respectively) to obtain spectra. Since such high ionic strengths were used in the NMR experiments, a correction for the activities of ammonia, R-5-P, and PRA was needed in order to determine an accurate equilibrium constant. Within the pH range studied (8.5–9.5), R-5-P and PRA were assumed to have the same charge; therefore, the activity coefficients for these two molecules were assumed to be identical. The correction for the activity coefficient of ammonia was made by changing the equation from K_{eq} = [PRA]/[R-5-P][NH_3] to $K_{eq'}$ = [PRA][H^+]/[R-5-P][NH_4^+] and by using an activity coefficient for NH_4^+ obtained from the work of Hurlen (1979). This $K_{eq'}$ was then converted to K_{eq} by the following equation:

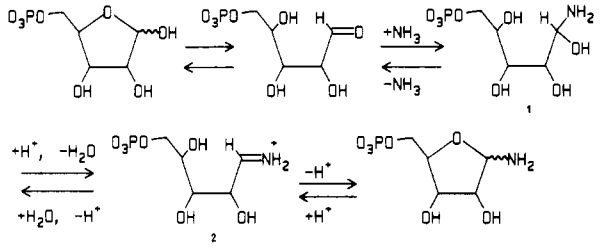


FIGURE 11: Scheme showing the proposed mechanism for the reaction of R-5-P and ammonia to form PRA.

$K_{eq} = K_{eq'}/K_{a(NH_3)}$. The $K_{a(NH_3)}$ was obtained by titration under identical conditions of ionic strength and temperature. The new equilibrium constant, calculated in this manner, is 1.7 ± 0.47 M^{-1}, which is in good agreement with the equilibrium constant obtained from k_f and k_d. Given the ionic strength differences between the GAR synthetase trapping experiments and NMR experiments, these values are in reasonably good agreement and are not tremendously different from the 0.7 M^{-1} (37 °C) originally reported by Nierlich and Magasanik.

On the basis of the above results, a mechanism for formation, decomposition, and anomerization of PRA can be proposed (Figure 11). This proposal is strongly influenced by the extensive mechanistic studies of Jencks and co-workers on the reaction of aldehydes and ketones with hydroxylamine, hydrazine derivatives, and amines (Jencks, 1964).

^{13}C NMR studies of Barker and co-workers indicate that, in solution at neutral pH, R-5-P predominantly exists as a 63.9:35.6 mixture of β-anomers: α-anomers with 0.1% of ring-opened aldehyde (Pierce et al., 1985). In addition, their saturation-transfer NMR experiments indicate that at this pH and 29 °C the ring-closed species is rapidly converted to the ring-opened sugar ($k_{obsd} \approx 15$–$25\ s^{-1}$). Thus ring-closed R-5-P is in rapid equilibrium with the ring-opened species. Ammonia is then proposed to attack the carbonyl of this ring-opened species to form carbinolamine **1**, which in an acid-catalyzed and perhaps rate-limiting step loses a molecule of H_2O to produce imine **2**. Ring closure would then occur to produce a mixture of α- and β-PRAs. This last step is proposed to be rapid, on the basis of the observation that α-PRA → β-PRA interconversion occurs at least 10000 times faster than hydrolysis and the assumption that both the anomerization and the hydrolysis reactions proceed through a common intermediate (**2**).

It is interesting to point out that the chemical properties of PRA differ strikingly from those of the more familiar and extensively studied pyranosyl amines (Isbell & Frush, 1958). Incubation of β-D-ribosylamine at pH 7.5 and room temperature under conditions similar to those of the ^{13}C NMR experiments with PRA indicate that after 1 h neither hydrolysis nor anomerization has occurred. In addition to the obvious chemical differences resulting from pyranose vs furanose ring systems, PRA is phosphorylated. This group may in fact catalyze the hydrolysis and/or anomerization of PRA. Intramolecular participation by phosphate has been proposed by Pierce et al. (1985) and Midelfort et al. (1976) to account for differences in rates of ring openings between furanosyl sugars and furanosyl sugar phosphates.

Determination of K_{eq} (using NMR and kinetic methods) reported in this paper provides the foundation for determination of the kinetic parameters for the GAR synthetase and PRPP amidotransferase reactions. These parameters are a prerequisite to addressing the intriguing possibility that PRA is channeled directly between PRPP amidotransferase and GAR synthetase in the purine pathway. Direct transfer would

avoid the problems with chemical instability of PRA extensively quantitated in this paper.

In addition, the anomerization studies suggest that GAR synthetase does not need to catalyze the anomerization of α- to β-PRA, the presumed substrate for the enzyme. The rate of anomerization, 27 s^{-1} (pH 8.5 and 37 °C), compares favorably with the rate of GAR synthetase turnover, 14 s^{-1} (pH 8.5, 37 °C). Rapid-quench experiments are presently being undertaken to show that as with other enzyme in the purine pathway (Caperelli et al., 1980; Schendel & Stubbe, 1986; Cheng et al., 1987) only the β-anomer binds and is turned over by the enzyme.

Registry No. α-PRA, 113158-36-4; β-PRA, 6062-06-2; [1-^{13}C]-R-5-P, 84446-92-4; [1-^{13}C]PRPP, 113056-58-9; [1-^{13}C]PRA, 113056-59-0; R-5-P, 4300-28-1; NH_3, 7664-41-7; PRPP synthetase, 9015-83-2; PRPP amidotransferase, 9031-82-7; D-[1-^{13}C]ribose, 70849-24-0; glycinamide ribonucleotide synthetase, 9032-01-3; ribokinase, 9026-84-0.

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