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Isotope Exchange as a Probe of the Kinetic Mechanism of Pyrophosphate-Dependent Phosphofructokinase†

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ABSTRACT: Data obtained from isotope exchange at equilibrium, exchange of inorganic phosphate against forward reaction flux, and positional isotope exchange of ^{18}O from the bridge position of pyrophosphate to a nonbridge position all indicate that the pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii* has a rapid equilibrium random kinetic mechanism. The maximum rates of isotope exchange at equilibrium for the [^{14}C]fructose 1,6-bisphosphate $\rightleftharpoons$ fructose 6-phosphate, [^{32}P]P_i $\rightleftharpoons$ $MgPP_i$, and Mg[^{32}P]PP_i $\rightleftharpoons$ fructose 1,6-bisphosphate exchange reactions increasing all four possible substrate–product pairs in constant ratio are identical, consistent with a rapid equilibrium mechanism. All exchange reactions are strongly inhibited at high concentrations of the fructose 6-phosphate (F6P)/P_i and $MgPP_i$/P_i substrate–product pairs and weakly inhibited at high concentrations of the $MgPP_i$/fructose 1,6-bisphosphate (FBP) pair suggesting three dead-end complexes, E:F6P:P_i, E:$MgPP_i$:P_i, and E:FBP:$MgPP_i$, in agreement with initial velocity studies [Bertagnolli, B. L., & Cook, P. F. (1984) *Biochemistry 23*, 4101]. Neither back-exchange by [^{32}P]P_i nor positional isotope exchange of ^{18}O-bridge-labeled pyrophosphate was observed under any conditions, suggesting that either the chemical interconversion step or a step prior to it limits the overall rate of the reaction.

Pyrophosphate-dependent phosphofructokinase (PP_i-PFK)[1] catalyzes the reversible phosphorylation of D-fructose 6-phosphate at the C-1 hydroxyl to give D-fructose 1,6-bisphosphate with magnesium pyrophosphate as the phosphoryl donor. The enzyme thus catalyzes the same chemical reaction as does the mammalian ATP-dependent phosphofructokinase. The enzyme from *Propionibacterium* exhibits Michaelis–Menten kinetics in both reaction directions at pH 8 and 25 °C (Bertagnolli & Cook, 1984).

The kinetic mechanism of the enzyme from *Propionibacterium freudenreichii* is proposed to be rapid equilibrium random with E:F6P:P_i, E:$MgPP_i$:P_i, and E:FBP:$MgPP_i$ dead-end complexes (Bertagnolli & Cook, 1984). However, initial velocity studies are not a good probe of the steps occurring within the interconversion of central complexes and indicate only that one or more steps responsible for the conversion of E:$MgPP_i$:F6P to E:Mg:P_i:FBP limit(s) the overall reaction.

The present study makes use of three different isotope exchange techniques. Isotope exchange at equilibrium is used to determine whether the mechanism is truly rapid equilibrium. Isotope exchange of [^{32}P]P_i back into pyrophosphate with the reaction running in the direction of production of P_i and FBP (exchange against the flow) and positional isotope exchange of ^{18}O-bridge-labeled pyrophosphate into the nonbridge position are used to determine whether steps other than phosphoryl transfer limit the interconversion of central complexes. These studies confirm the rapid equilibrium random mechanism proposed from initial velocity data and further suggest that the chemical step(s) probably limit(s) the interconversion of central complexes.

Materials and Methods

Enzymes. Pyrophosphate-dependent phosphofructokinases from *Propionibacterium freudenreichii* and *Phaseolus aureus* were both obtained in crude form from Sigma and purified according to Bertagnolli and Cook (1984) and Bertagnolli et al. (1986). Fructose-1,6-bisphosphate aldolase, triosephosphate isomerase, phosphoglucose, isomerase, and α-glycerophosphate dehydrogenase from rabbit muscle, glucose-6-phosphate de-

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[1] Abbreviations: PP_i-PFK, inorganic pyrophosphate dependent phosphofructokinase; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; Taps, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TEA, triethylamine.

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hydrogenase, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase from yeast were obtained from Sigma and tested as discussed previously (Bertagnolli & Cook, 1984) for contaminating activities.

Chemicals. Sodium pyrophosphate decahydrate, F6P, FBP, NAD, NADH, fructose 2,6-bisphosphate, ADP, and Taps were from Sigma. D-Glyceraldehyde 3-phosphate was prepared from its diethyl acetal (Sigma). Approximately 50 mg of the diethyl acetal was dissolved in 2 mL of water, and 2 mL of Dowex 50W-X8 in the H^+ form was added. The solution was submerged in boiling water for 30 s and then quickly cooled, and the resin was removed by filtration. The resulting solution was either used immediately or stored at −20 °C for later use.

The potassium salt of [U-^{14}C]fructose 1,6-bisphosphate (239 mCi/mmol) and the dipotassium salt of inorganic [^{32}P]-phosphate (200 mCi/mmol) were from Amersham. Tetrasodium [^{32}P]pyrophosphate (8.27 μCi/mmol) was obtained from Dupont NEN Research.

Substrate concentrations were calibrated via complete conversion of reactant to product with the PP_i-PFK reaction and the aldolase/triosephosphate isomerase/glycerolphosphate dehydrogenase couple for F6P (with 1 mM $MgPP_i$) and $MgPP_i$ (with 2 mM F6P) by use of the change in NADH concentration reflected by the change in absorbance at 340 nm to calculate initial substrate concentration. In the case of FBP and P_i, calibrations again made use of the PP_i-PFK reaction but with the phosphoglucose isomerase/glucose-6-phosphate dehydrogenase couple, 1 mM Mg^{2+}, and 1 mM FBP (for P_i) or 5 mM P_i (for FBP) monitoring the appearance of NADPH. All substrate calibrations were carried out at least in duplicate. In all cases correction was made for the concentration of metal chelate complexes as described previously (Bertagnolli & Cook, 1984).

Isotope Exchange at Equilibrium. Rates of isotopic exchange at equilibrium were measured for the F6P $\rightleftharpoons$ FBP, P_i $\rightleftharpoons$ PP_i, and PP_i $\rightleftharpoons$ FBP exchange reactions, varying in each case all four reactant/product pairs in constant ratios. Generally, two incubation mixtures were prepared that contained the following in a volume of 0.6 mL: 100 mM Taps, pH 8; 0.11 mM F6P; 0.09 mM $MgPP_i$; 0.1 mM FBP; 0.36 mM P_i; 1 mM Mg^{2+} (a given substrate/product pair was then changed as indicated under Results). To one of the above was added 0.0025 unit of PP_i-PFK, and the mixture was incubated for 3 h at 25 °C while the remaining incubation mixture served as a control. After equilibrium was attained, a 10-μL volume of radioactive reactant was added to each of the above pairs so that they contained 80 000 cpm. Aliquots of 0.15 mL were taken at 4, 8, 12, and 16 min and added to 0.15 mL of 200 mM EDTA to quench the reaction. Reactants were then fractionated as discussed below on a Bio-Rad anion-exchange column via HPLC. Radioactive fractions were pooled, and a 1-mL aliquot was added to 10 mL of Aquascint scintillation cocktail for counting ^{14}C or ^{32}P with a Beckman Model LS 3801 scintillation counter.

Separation of reactants via HPLC with a Bio-Rad Aquapore anion-exchange column (250 × 4.6 mm, 7-μm pore size) was carried out with a mixture 20 mM in each of the reactants in 100 mM Taps, pH 8. A 0.1-mL sample was applied to the column, which was developed with a linear gradient (0–1 mM NaCl in 100 mM Taps, pH 7.7), and each run took ca. 30 min. Reactant positions were located by use of substrate calibration assays as discussed above under the paragraph on substrate concentration calibration. The reactants were eluted in the order of F6P, P_i, FBP, and PP_i. In all cases except FBP and PP_i, base-line separation was achieved. The lack of base-line separation was only a problem with the $MgPP_i$ $\rightleftharpoons$ FBP exchange reaction, and in this case the amount of [^{32}P]P_i produced with time was taken to be equal to the amount of [^{32}P]FBP produced. To test this latter assumption, complete conversion of [^{32}P]$MgPP_i$ to [^{32}P]P_i and [^{32}P]FBP gave essentially identical counts in each.

In addition to the above, contamination of the radioactive reactants was tested by running a sample of the reactant on HPLC. In the case of [^{14}C]FBP and [^{32}P]P_i, no detectable [^{14}C]F6P and [^{32}P]PP_i were observed. In the case of [^{32}P]PP_i, a 0.8% contaminant of [^{32}P]P_i was detected, and all reaction mixtures were corrected for this minor contaminant. The percentage recovery in all cases based on applied cpm was ≥96%, while the percentage recovery of reactant applied was ≥95%.

Nonequilibrium Isotope Exchange (Exchange against the Flow) with [^{32}P]P_i. In order to determine whether inorganic phosphate could be incorporated into $MgPP_i$ under conditions in which the net flux of the phosphofructokinase reaction is in the direction of F6P phosphorylation, the following experiment was carried out. The reaction mixture included the following in a 1-mL volume: 10 mM NH_4HCO_3, pH 8; 10.4 mM $MgCl_2$; 10 mM pyrophosphate; 10.04 mM F6P; 9 mM [^{32}P]P_i (2×10^6 cpm). Five separate reaction mixtures were set up, and enzyme (0.1 unit) was added to three at the same time ($t = 0$). One of the reaction mixtures served as a minus enzyme control at time zero and was analyzed as stated below for separation of P_i and PP_i after addition of 200 μmol of EDTA. The reactions that included enzyme were terminated at 30, 60, and 90 min by the addition of 200 μmol of EDTA. This EDTA concentration was shown with the coupled spectrophotometric assay above to be sufficient to stop the reaction. After termination of the reaction, P_i and PP_i were separated as stated below. Finally, once the final reaction was terminated (90 min), EDTA was added to the second minus enzyme control followed by separation of P_i and PP_i as stated below.

The separation of P_i and PP_i made use of DEAE-Sephadex A-50 in Econo columns (Bio-Rad) with a modification of the method of Gray (1971). The DEAE (≃10 g) was swollen in 1 L of 1 M NH_4HCO_3, pH 8, for 24 h at room temperature. The mixture was decanted, 20 mM NH_4HCO_3 was added, and this procedure was repeated several times. The column was then washed with 10 column volumes (20 mL) of 20 mM NH_4HCO_3 (pH 7.5–8.0).

After the reaction was terminated with EDTA, the entire reaction mixture was applied and allowed to wash through followed by an additional 6.8 mL of 20 mM NH_4HCO_3. The wash was followed by 6.5 mL each of 80 mM and 160 mM NH_4HCO_3. Final volumes of each of the fractions were recorded, and a 1-mL aliquot of each fraction was added to 9 mL of Aquascint scintillation cocktail and counted for 1 min. In addition to the minus enzyme controls and time points, an amount of [^{32}P]P_i equal to the total amount per experiment was counted. In every experiment, the sum of the counts for the three washes was equal to the total amount added. Thus, chemical quenching was not a problem. The concentration of PP_i in each fraction was also determined by use of PP_i-PFK at high concentrations (1 unit/mL) and coupling the production of FBP to the aldolase/triosephosphate isomerase/α-glycerophosphate dehydrogenase reaction as described above.

Positional Isotope Exchange Experiment (PIX). The ^{18}O-bridge-labeled pyrophosphate was synthesized according to Reynolds et al. (1983). Quantitation of ^{18}O-bridge-labeled, ^{18}O-nonbridge-labeled, and unlabeled pyrophosphate was ob-

tained by use of the relative peak areas from the ^{31}P NMR spectra (collected at 97.6 and 202.45 MHz) as representative of the relative enrichments. From a ^{31}P NMR spectrum generated at 97.6 MHz, the percentage of bridge-labeled, nonbridge-labeled and unlabeled species corresponded to 73.1%, 10%, and 16.9%, respectively. At 202.5 MHz, an ATP sample was used with the percentages corresponding to 69.2%, 14.5%, and 16.3%, respectively. Analysis at 97.6 MHz made use of peak intensities, while at 202.5 MHz analysis was carried out with peak areas calculated with the GEMCAP curve analysis deconvolution routine contained in the Nicolet 1280 software package.

For turnover experiments the reaction mixtures contained the following in a 30-mL final volume: Taps, pH 8, 100 mM; [^{18}O]pyrophosphate, 10 mM; F6P, 10 mM; $MgCl_2$ (or $CaCl_2$), 1 mM. All reactant concentrations (except metal pyrophosphate) were corrected for the amount of the metal–chelate complex present as described above. From each 30-mL reaction mixture, a 3-mL aliquot was removed to monitor the reaction progress. To monitor FBP concentration, the following were added to 1 mL of the above 3-mL aliquot: PP_i-PFK, 0.05 unit; FBP aldolase, 13 units; α-glycerophosphate dehydrogenase, 2.5 units; triosephosphate isomerase, 25 units; NADH, 0.2 mM. To monitor P_i concentration, the following were added to 1 mL of the above 3-mL aliquot: PP_i-PFK, 0.05 unit; glyceraldehyde-3-phosphate dehydrogenase, 7 units; phosphoglycerate kinase, 7 units; NAD, 0.3 mM; ADP, 0.3 mM. In the former case, the decrease in 340 nm absorbance was monitored while in the latter the increase in 340 nm absorbance was monitored. Both monitors of the reaction were used for all experiments, and in all cases the two monitors agreed well.

The remaining 27 mL of cocktail was then incubated in a water bath at 25 °C, and to this was added 0.05 unit/mL PP_i-PFK. The incubation mixture was then allowed to react for variable amounts of time depending upon the rate of turnover. Incubation times varied from as short as 4 h to as long as 42 h. Total turnover was determined by taking a 5–20-μL sample of the quenched assay mixture and performing end-point analysis with the aldolase–glycerophosphate dehydrogenase–triosephosphate isomerase assay. Turnover varied from as little as 10% (using Ca^{2+} and enzyme from *P. freudenreichii*) to as much as 57%. All experiments in which the metal ion was changed were performed only with enzyme from *P. freudenreichii* while, in the case of the experiment where Mg^{2+} was the divalent metal, enzyme from *Ph. aureus* was also used in separate experiments. After reaction, samples for determination of reactant concentration were quenched by quick freezing and were stored for later analysis. The remainder of the 27-mL cocktail was diluted with cold distilled deionized H_2O (200 mL), the pH was adjusted to 8, and the entire contents were loaded onto a DEAE-Sephadex A-25 anion-exchange column (Pharmacia) in the HCO_3 form. The column was developed with a 800-mL linear (0.07–0.7 M) gradient of TEA-HCO_3, pH 8. Fractions of 30 mL were collected with a Gilson FC-100 fraction collector and tested for P_i and PP_i according to the method of Ames (1966). Fractions containing pyrophosphate (eluting between approximately 0.35 and 0.4 M) were then pooled and concentrated in vacuo to a dry powder. Absolute methanol was then repeatedly added and the dissolved mixture dried in vacuo to remove all remaining traces of TEA-HCO_3. The remaining film lining the bottom of the round-bottom flask was then dissolved with 3 × 1 mL washes of absolute methanol, and the washes were pooled in a 30-mL Corex centrifuge tube. The product was then converted to the Na^+ salt by precipitation with the dropwise addition of 1 N NaI/acetone. The flocculent white precipitate was then centrifuged at 6000 rpm with a Sorvall SS-34 rotor. The white pellet was then resuspended with absolute acetone and recentrifuged at least 4 times until only a clean white pellet remained. The pellet was then dissolved in a minimal amount of distilled deionized H_2O. The pH was adjusted to 11.5–12 with 1 N NaOH, and absolute ethanol was added slowly in a dropwise fashion until fine needles began to appear. The solution was then refrigerated approximately 1–2 h. The crystalline pyrophosphate was then suction filtered, washed with ethanol, and stored.

NMR Spectra. Samples for the NMR were prepared in 0.6 mL of 100 mM Hepes, pH 8. Samples were then diluted to 10 mL with distilled/deionized water and passed through Chelex-100 cation-exchange resin twice. Strict attention was paid to making all glassware metal-free by initially soaking in 50% (v/v) H_2SO_4–HNO_3 followed by washing with distilled/deionized H_2O. After passage through Chelex, samples were lyophilized, and the preparation was reconstituted with 0.6 mL of 33% HOD (Aldrich, low paramagnetic, T_1 = 48.1 s).

NMR spectra were run at 97.57 MHz (5.6 T), with a sweep width of ±500 Hz and a 4.09-s acquisition time. Spectra were digitized with 8192 data points, producing a digital resolution of 0.0026 ppm/data point. Resolution to 0.00065 ppm/data point was obtained twice as a result of zero filling. Typically, 200–1000 acquisitions were required to yield signal to noise ratios ≥90. Spectra were resolved to ±2.2%. Apodization by double-exponential multiplication (DM) was utilized to enhance resolution of spectra. NMR spectra were also generated at 202.5 MHz (11.6 T), utilizing a sweep width of ±1000 Hz and a spectral size of 16 384 data points. Acquisition time was 4.09 s with a 3-s delay. Digital resolution was identical with that attained at 97.57 MHz both prior to and after zero filling. Typically, 200–1000 acquisitions were required to yield signal to noise ratios ≥127.4. Spectra were resolved to ±1.6%.

Data Processing. Rates of isotope exchange were calculated according to eq 1 (Purich & Allison, 1980), where V_{ex} is the

$$V_{ex} = -\frac{AC}{A + C}\frac{\ln (1 - f)}{t[\text{enzyme}]} \quad (1)$$

initial velocity of isotope exchange, A and C are the equilibrium amounts of the exchanging species in moles, t is the time in minutes of the exchange reaction, and f is the fractional attainment of isotopic equilibrium at time t. The value of f was calculated by obtaining the ratio of the fraction of radioactivity as A at time t and after attainment of isotopic equilibrium.

The reciprocal of the exchange rate was plotted against the reciprocal of one of the reactants of a reactant/product pair raised in constant ratio. Data were fitted with the appropriate rate equation and FORTRAN programs developed by Cleland (1979). Data adhering to a linear reciprocal plot were fitted with eq 2 while those exhibiting substrate inhibition were fitted with eq 3. In eq 2 and 3, v is the initial rate of isotope

$$v = \frac{VA}{K_a + A} \quad (2)$$

$$v = \frac{VA}{K_a + A + A^2/K_I} \quad (3)$$

exchange, V is the maximum velocity of the exchange reaction, K_a is the concentration of the varied substrate/product pair that gives half the maximum exchange rate, and K_I is the substrate inhibition constant for a substrate/product pair.

Table I: Kinetic Parameters from Isotope Exchange at Equilibrium

parameter[a]	variable substrate/product pair			
	F6P/FBP	F6P/P_i	$MgPP_i$/FBP	$MgPP_i$/P_i
	Reverse Reaction: [^{14}C]FBP ⇌ F6P			
V_{max} (mM min^{-1} μg^{-1})	85 ± 2	76 ± 2	73 ± 2	100 ± 4
K_m (mM)	0.093 ± 0.005	0.080 ± 0.007	0.060 ± 0.004	0.120 ± 0.010
K_I (mM)		4.7 ± 0.7	≥9.4	3.3 ± 0.5
	Reverse Reaction: [^{32}P]P_i ⇌ $MgPP_i$			
V_{max} (mM min^{-1} μg^{-1})	94 ± 1	89 ± 2	75 ± 2	92 ± 2
K_m (mM)	0.052 ± 0.002	0.048 ± 0.005	0.012 ± 0.002	0.041 ± 0.002
K_I (mM)		5.0 ± 0.7	≥32.5	2.6 ± 0.2
	Forward Reaction: [^{32}P]$MgPP_i$ ⇌ FBP			
V_{max} (mM min^{-1} μg^{-1})	105 ± 5	120 ± 12	89 ± 8	110 ± 19
K_m (mM)	0.28 ± 0.03	0.32 ± 0.05	0.18 ± 0.03	0.22 ± 0.06
K_I (mM)		0.98 ± 0.18	≥5.0	0.71 ± 0.21

[a] The K_m for F6P when either F6P/FBP or F6P/P_i is increased in constant ratio or for $MgPP_i$ when either $MgPP_i$ or $MgPP_i$/P_i is increased in constant ratio.

The amount of ^{18}O-bridge-labeled pyrophosphate exchanged was calculated according to (Litwin & Wimmer, 1979)

$$[^{18}O]PP_i \text{ exchanged} = \frac{X}{\ln (1 - X)}[PP_i]_0[\ln (1 - f)] \quad (4)$$

where X is the fraction of pyrophosphate converted to product and $[PP_i]_0$ is the original concentration of PP_i added. The term f is equal to $(P_t - P_0)/(P_D - P_0)$, where P_t, P_0, and P_D refer to the percentage of ^{18}O-nonbridge-labeled PP_i present at time t, time zero, and equilibrium. The bridge label can scramble into any one of three positions (assuming torsional symmetry). At 97.6 MHz, $P_0 \simeq 10\%$, $P_D \simeq 55.4\%$, and P_t is the percentage of nonbridged PP_i present at time t while at 202.5 MHz the values of P_D and P_0 are 55.8% and 14.5%, respectively.

Results

Isotope Exchange at Equilibrium. Plots of $-\ln (1 - f)$ vs time were linear for all exchange reactions over the entire time scale used. In addition, the rates of all exchange reactions are a linear function of the concentration of enzyme over the range of enzyme concentration used.

The rates of isotope exchange at equilibrium were obtained for the [^{32}P]$MgPP_i$ ⇌ FBP, [^{14}C]FBP ⇌ F6P, and [^{32}P]P_i ⇌ $MgPP_i$ exchange reactions as a function of increasing constant ratio. Rates for the following substrate/product pairs[2] were measured: F6P/FBP, $MgPP_i$/FBP, F6P/P_i, and $MgPP_i$/P_i. Data are shown in Figure 1 (A–D) for the three exchange reactions, increasing a given substrate/product pair. It should be noted that even though the $MgPP_i$ ⇌ FBP exchange rate is obtained from the opposite reaction direction, it can be compared to the exchange reactions obtained in the other direction since the maximum velocities in the two reaction directions are equal (Bertagnolli & Cook, 1984). No substrate inhibition of the exchange reaction is observed for all three exchange reactions when F6P/FBP is increased in constant ratio, Figure 1A. Data were collected up to 2.2 mM F6P for experiments shown in Figure 1A and 1.8 mM $MgPP_i$ for experiments shown in Figure 1B. The two highest concentration points in the case of the exchanges in which

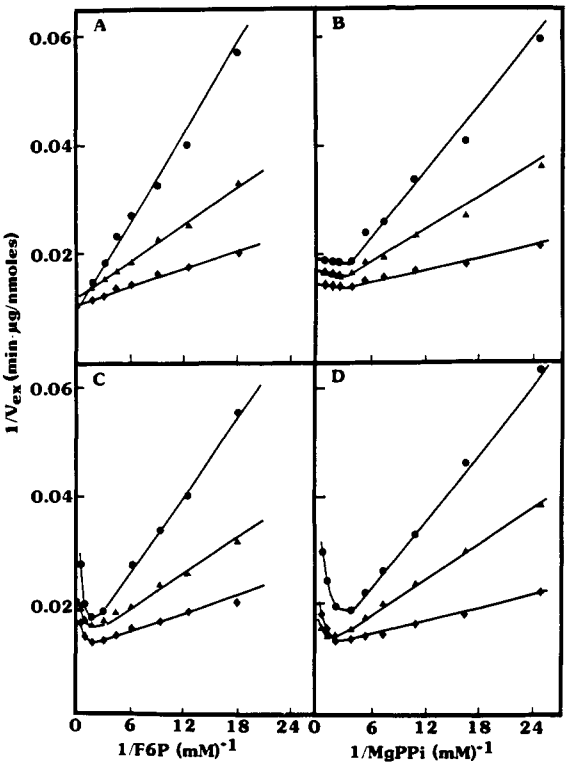

FIGURE 1: Double-reciprocal plots of the initial velocity of isotope exchange at equilibrium for the [^{32}P]$MgPP_i$ ⇌ FBP (●), [^{14}C]FBP ⇌ F6P (▲), and [^{32}P]P_i ⇌ $MgPP_i$ (♦) exchange reactions at pH 8, 100 mM Taps, and 25 °C. Exchange rates are measured at an increasing concentration of one reactant maintained in constant ratio with a product: (A) varying concentrations of F6P maintained in a ratio of 1.1 with FBP; (B) varying concentrations of $MgPP_i$ maintained in a ratio of 0.9 with FBP; (C) varying concentrations of F6P maintained in a constant ratio of 0.3 with P_i; (D) varying concentrations of $MgPP_i$ maintained in a constant ratio of 0.25 with P_i. The concentrations of all other reactants are as indicated under Materials and Methods. Solid curves are theoretical from fits using eq 2 (A and B) and 3 (C and D) while points are experimental.

$MgPP_i$/FBP was increased in constant ratio were slightly substrate inhibited, and these points were eliminated for computer fitting. An estimate of the K_I for substrate inhibition in this case is greater than 5.0 mM. Values are included in Table I. All exchange reactions exhibit substrate inhibition when either F6P/P_i or $MgPP_i$/P_i is increased in constant ratio, Figure 1C,D. Quantitative data are shown in Table I.

Nonequilibrium Exchange of [^{32}P]P_i and $MgPP_i$. By use of saturating $MgPP_i$ and F6P with [^{32}P]P_i equal to its dissociation constant as a product inhibitor, no significant radio-

[2] The [^{14}C]FBP ⇌ F6P and [^{32}P]P_i ⇌ $MgPP_i$ exchange rates should be plotted by convention against the reciprocal of FBP and P_i concentrations and not F6P and $MgPP_i$ concentrations as shown in Figure 1. However, the maximum velocity will not change dependent on which of the above is plotted, and the K_m and K_I values are only used as indicators of similarity in the data. As a result, we chose to plot all three exchange rates in the same figure with F6P and $MgPP_i$ as substrates.

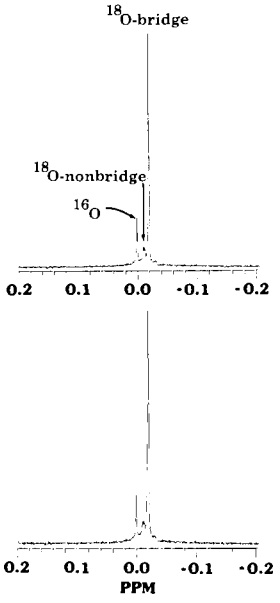

FIGURE 2: ^{31}P NMR spectra at 202.5 MHz of the phosphoryl resonances of [^{18}O]pyrophosphate. Conditions are as described under Materials and Methods with the enzyme from *P. freudenreichii*. Upper and lower spectra are from 4-h incubations minus and plus enzyme, respectively.

activity was detected in the $MgPP_i$ up to 90 min after the addition of enzyme. There was a small amount (ca. 5%) of residual radioactivity detected in the PP_i fraction from the DEAE column as a result of tailing of the P_i peak. However, the total amount of counts in this case is identical with the control value.

Positional Isotope Exchange. A ^{31}P NMR spectrum (at 202.5 MHz) for bridge-labeled [^{18}O]pyrophosphate incubated in the absence and presence of PP_i-PFK from *P. freudenteichii* is shown in Figure 2. As can be seen, there is little or no difference in bridge and nonbridge intensities for the plus enzyme spectrum compared to those of the control. Identical results were obtained with the enzyme from *Ph. aureus* in the presence and absence of a 1 μM concentration of the activator, fructose 2,6-bisphosphate (data not shown).

It should be noted that in the spectra taken at 202.5 MHz, Figure 2 for example, the nonbridge peak is split into an apparent doublet. This is a result of the ^{18}O in the nonbridge position imparting a small but discernible amount of nonequivalence compared to the ^{31}P with only ^{16}O. Thus, the inner peaks of an AB pattern are observed. This phenomenon has been observed previously by Marschner et al. (1983) at 202.5 MHz where the inner peaks are resolved. Integrating the areas under the curves, one calculates no significant change in the amount of ^{18}O-nonbridge-labeled pyrophosphate in the presence of enzyme. Extrapolating from the confidence limits of the spectra generated (S/N = 127.4 at 202.5 MHz), an upper limit on the rate of partitioning back to substrates from the central complex vs partitioning to products at saturating concentrations can be obtained. For a fractional turnover of 0.57, values of <0.026 (202.5 MHz) and <0.042 (97.5 MHz) are estimated for the exchange/turnover ratio. Experiments carried out with the *Ph. aureus* enzyme give a value of about 0.05 for a fractional turnover of 0.29. Some of the experiments for the latter enzyme were carried out at a lower field strength of 97.5 MHz for which a S/N of 90 was obtained.

Attempts to induce PIX were undertaken by replacing Mg^{2+} with Ca^{2+} (data not shown) after the work of von der Saal et al. (1985) on GMP synthetase. With the metal substitution it should be noted that only 10% turnover was obtained in 42 h. Experiments were also carried out at pH 6.5 (data not shown). In all cases and under any given set of conditions, PIX was never observed.

Discussion

Isotope Exchange at Equilibrium. The maximum rates of isotope exchange at equilibrium for all three exchange reactions, increasing any of the four substrate/product pairs in constant ratio, are similar with an average value of 93 ± 8 mM min^{-1} μg^{-1}. In addition, when the fixed substrate/product pair remains the same from one exchange reaction to another, the slopes of the double-reciprocal plot are very similar for the exchange reaction obtained in the same reaction direction as they should be for a rapid equilibrium random mechanism (Purich & Allison, 1982). From initial velocity studies, Bertagnolli and Cook (1984) also suggested that three dead-end complexes were allowed, that is, E:F6P:P_i, E:$MgPP_i$:P_i, and E:FBP:$MgPP_i$. In the present case, all isotope exchange reactions are strongly inhibited at high concentrations of the F6P/P_i and $MgPP_i$/FBP pairs in agreement with the previous report. In addition, slight substrate inhibition was observed in the case of the higher levels of the $MgPP_i$/FBP pair.

The V_{max} values for all exchange reactions are remarkably similar but particularly for the two exchanges carried out in the direction of P_i phosphorylation. In addition, the F6P and $MgPP_i$ K_m values are similar for the FBP $\rightleftharpoons$ F6P and P_i $\rightleftharpoons$ $MgPP_i$ exchange reactions as are the K_I values measured for increasing F6P/P_i and $MgPP_i$/P_i pairs. For the $MgPP_i$ $\rightleftharpoons$ FBP exchange, the K_m values are virtually identical for F6P and $MgPP_i$ when F6P/FBP and F6P/P_i and when $MgPP_i$/FBP and $MgPP_i$/P_i, respectively, are increased.

As a result, the isotope exchange at equilibrium experiments independently predicts a rapid equilibrium random mechanism with three dead-end complexes in agreement with initial velocity data (Bertagnolli & Cook, 1984).

Positional Isotope Exchange. The rapid equilibrium random mechanism proposed from initial velocity studies and confirmed with the isotope exchange studies presented in this paper indicate only that the interconversion of E:$MgPP_i$:F6P and E:Mg:P_i:FBP is rate determining. These results provide no evidence, however, on whether the chemical interconversion step in which a phosphoryl moiety is transferred to the 1-hydroxyl of F6P is rate determining. This is true since there may exist either rate-determining or partially rate determining conformational changes, required to bring the enzyme to a closed catalytic conformation upon binding of $MgPP_i$ and F6P and subsequently to reopen the catalytic site once catalysis has taken place. One way of potentially detecting a slow step after catalysis in the case of kinases is the use of positional isotope exchange. With this technique, one looks for the scrambling of isotope from the bridge oxygen of pyrophosphate to the nonbridge oxygen of pyrophosphate after reaction has proceeded for a finite number of turnovers. If an increase in the ^{18}O content in the nonbridge position has occurred (compared to control), this may have occurred for one of two reasons. First, release of products after catalysis is slow, and there is time for pyrophosphate to be re-formed and released. Second, inorganic phosphate may be released and may then rebind to the E:FBP complex, allowing the reverse reaction to occur, thus forming pyrophosphate. In the latter case one would expect back-exchange of [^{32}P]P_i into PP_i as the reaction is run in the direction of FBP and P_i. In the present case, exchange against the flow was not observed, hence, any positional isotope exchange observed must be a result of slow release of both products. However, under all conditions tested PIX was not obtained. Raushel and Garrard (1984) have shown that in

some cases PIX can be induced with product inhibition by the nonlabeled product. In the present case, however, dead-end complexes are also formed, and conditions required to give sufficient turnover would require large volumes (2–3 L), making these experiments impractical.

There are two possible means of explaining the lack of positional isotope exchange:

(1) Once catalysis has taken place, the dissociation of the products is appreciably faster than the net rate of catalysis and product release in the reverse direction. This would require that either the chemical steps themselves or some step (e.g., conformational change) prior to the chemical step in the forward direction be rate determining. Either of these two would be consistent with a rapid equilibrium random mechanism.

(2) Catalysis may occur and the enzyme may not provide enough freedom to allow a free rotation about the O–P bond so that the oxygens in the intermediate are not torsionally symmetric. In other words, the oxygens of phosphate are tightly bound. This is rather unlikely since molecular orbital calculations have shown this barrier to rotation, at least free of enzyme, to be very low ($\Delta G^{\ddagger} \simeq 1$ kcal, $k_{rot} = 10^{12}$ s^{-1}) (Engelke, 1973). However, this possibility cannot be strictly ruled out, particularly since there is very likely interaction of both nucleophile and leaving group with Mg^{2+} (Herschlag & Jencks, 1987). In this case, the back-reaction would occur prior to release of products, but PIX would not be observed.

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Nitrogen Isotope Effects on Glutamate Decarboxylase from *Escherichia coli*†

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ABSTRACT: The nitrogen isotope effect on the decarboxylation of glutamic acid by glutamate decarboxylase from *Escherichia coli* has been measured by comparison of the isotopic composition of the amino nitrogen of the product γ-aminobutyric acid isolated after 10–20% reaction with that of the starting glutamic acid. At pH 4.7, 37 °C, the isotope effect is $k^{14}/k^{15} = 0.9855 \pm 0.0006$ when compared to unprotonated glutamic acid. Interpretation of this result requires knowledge of the equilibrium nitrogen isotope effect for Schiff base formation. This equilibrium isotope effect is $K^{14}/K^{15} = 0.9824$ for the formation of the unprotonated Schiff base between unprotonated valine and salicylaldehyde. Analysis of the nitrogen isotope effect on decarboxylation of glutamic acid and of the previously measured carbon isotope effect on this same reaction [O'Leary, M. H., Yamada, H., & Yapp, C. J. (1981) *Biochemistry 20*, 1476] shows that decarboxylation and Schiff base formation are jointly rate limiting. The enzyme-bound Schiff base between glutamate and pyridoxal 5′-phosphate partitions approximately 2:1 between decarboxylation and return to the starting state. The nitrogen isotope effect also reveals that the Schiff base nitrogen is protonated in this intermediate.

Most amino acid decarboxylases require pyridoxal 5′-phosphate (PLP)[1] as a cofactor. The generally accepted mechanism for this reaction is shown in Scheme I. The first step is substrate binding. The second step is Schiff base interchange, in which the nitrogen of the enzyme–PLP Schiff base is replaced by the amino nitrogen of the substrate. This is a multistep process involving numerous proton transfers. The third step is the actual decarboxylation step, in which the protonated pyridine nitrogen serves as an electron sink. Stereochemical and conformational control are principally manifested in this step (O'Leary & Piazza, 1978). All available evidence indicates that this step is irreversible (O'Leary et al., 1981). Steps corresponding to k_7 and k_9 involve Schiff base interchange and release of the decarboxylated product. Protonation of the decarboxylated intermediate usually occurs with retention of configuration at the α-carbon atom (Dunathan, 1971; Floss & Vederas, 1982). Steps following decarboxylation are presumably of little kinetic significance and are

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; GABA, γ-aminobutyric acid; P_i, inorganic phosphate.

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