Unambiguous Stereochemical Course of Rabbit Liver Fructose Bisphosphatase Hydrolysis†

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ABSTRACT: The stereochemical course of rabbit liver fructose bisphosphatase (EC 3.1.3.11) was determined by hydrolyzing the substrate analogue (S_P)-[1-^{18}O]fructose 1-phosphorothioate 6-phosphate in $H_2{}^{17}O$, incorporating the chiral, inorganic phosphorothioate product into adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS), and analyzing the isotopic distribution of ^{18}O in ATPβS by ^{31}P NMR. The result indicates that the 1-phosphoryl group is transferred with inversion of configuration. A series of single-turnover experiments ruled out an acyl phosphate intermediate in the hydrolysis. Consequently, fructose bisphosphatase catalyzes the hydrolysis of fructose 1,6-bisphosphate via a direct transfer of the phosphoryl moiety to water.

Fructose 1,6-bisphosphatase (EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase, FBPase)[1] catalyzes the hydrolysis of D-fructose 1,6-bisphosphate (Fru-1,6-P_2) to D-fructose 6-phosphate (Fru-6-P_1) and orthophosphate (P_i) (Rahil et al., 1982) with cleavage of the P–O bond (Pontremoli et al., 1965). Several of our studies have attempted to determine whether a phosphoryl enzyme (E–P intermediate) (Benkovic et al., 1979; Rahil et al., 1982; Sharp & Benkovic, 1979) is involved in the hydrolysis but did not rule out such a steady-state species.

The present study employs a stereochemical probe as a means of determining the mechanistic course of the hydrolysis. Toward this end, a chiral substrate analogue, (S_P)-D-fructose 1-[^{18}O]thiophosphate 6-phosphate [(S_P)-[^{18}O]FBPS], was synthesized. The enzymatic hydrolysis of this substrate analogue in $H_2{}^{17}O$ resulted in the formation of chiral [^{17}O,^{18}O]phosphorothioate, (chiral SP_i). The chirality of this product was determined by a modification of Webb & Trentham's (1980) procedure revealing that the stereochemical course of the hydrolysis was one of inversion.

The interpretation of this stereochemical result presumes that if a covalent E–P intermediate was involved, its subsequent hydrolysis would involve water attack on the carbon atom. The second part of this study is concerned with determining this assumption's validity. Gas chromatography–mass spectroscopy was employed to analyze the isotopic enrichment of P_i from a series of single-turnover experiments in $H_2{}^{18}O$ in order to establish if direct nucleophilic attack by water occurs on the 1-phosphate.

Experimental Procedures

Materials. Rabbit liver FBPase was prepared as described by Benkovic et al. (1976). Acetate kinase (*Escherichia coli*), aldolase (rabbit muscle), alkaline phosphatase (*E. coli*), fructose-6-phosphate kinase (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), lactic dehydrogenase (rabbit muscle), myokinase (rabbit muscle), phosphoglycerate kinase (yeast), pyruvate kinase (rabbit muscle), and triosephosphate isomerase (rabbit muscle) were from Sigma. All the enzymes except alkaline phosphatase and pyruvate kinase were dialyzed against 4 L of 20 mM Tris-HCl buffer, pH 8.0, 1 mM DTE, and 1 mM EDTA at 5 °C for 24 h. ADP (sodium salt), AMP (sodium salt), Fru-1,6-P_2 (sodium salt), Fru-6-P (sodium salt), and NAD (yeast) were from Sigma. DCQ was from Fisher, and *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine was from Aldrich. Biobead SX-1 (200–400 mesh), Dowex AG 1-X8 (200–400 mesh), and Chelex 100 (200–400 mesh) were from Bio-Rad. Sephadex G-25 (coarse) and DEAE-Sephadex A-25 were from Pharmacia. The 10% Silar 10-C on Gas Chrom Q (100–200 mesh) was from Supelco. PEI–cellulose thin-layer plates were from E. Merck, and silica gel thin-layer plates were from Eastman Kodak. TEAB (1 M) was prepared by bubbling carbon dioxide through a sintered glass bubbler into a 5 °C aqueous solution of 1 M triethylamine until the pH reached 7.5. The triethylamine was freshly distilled from calcium hydride. All fraction-collecting tubes were new and P_i free. All glassware was soaked for 8 h in an KOH-saturated/2-propanol bath, rinsed with water, soaked for 8 h in 50% H_2SO_4, and finally rinsed with water. All other organic reagents, buffers, and inorganic salts were commercially available reagent-grade chemicals. Doubly distilled deionized water was used throughout.

Analytical Procedure. Concentration measurements were performed on a Gilford Model 240 spectrophotometer; pH measurements were made on a Radiometer Model 22 pH meter equipped with a Model PHA 630-Pa scale expander at 25 °C. TLC system A was PEI cellulose plates developed with 0.75 M KH_2PO_4 (pH 3.55); TLC system B was silica gel plates developed with 2-propanol–ammonium hydroxide–water (6:3:1). UV-absorbing compounds were visualized with UV light (254 nm); sulfur-containing compounds were detected with the sulfur-sensitive reagent DCQ (1% in acetic acid). The thiophosphate monoesters were visualized as yellow and the diesters as pink, and SP_i was visualized as brown spots. DEAE Sephadex A-25 was converted to the bicarbonate form by batch washing with 20 bed volumes of 1 M TEAB. In all

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DCQ, 2,6-dibromo-*N*-chlorobenzoquinonimine; DEAE, diethylaminoethyl; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; NAD, β-nicotinamide adenine dinucleotide; PEI, poly(ethylenimine); TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; IU, international unit (1 μmol min^{-1} mg^{-1} at 25 °C); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate).

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cases, a 2.5 cm × 26 cm column was utilized and preequilibrated with the starting elution buffer. A flow rate of 1.3 mL/min was maintained, and 16–20-mL fractions were collected. DEAE-Sephadex A-25 and Sephadex G-25 column elutions were monitored spectrophotometrically at 260 and 280 nm, respectively. Biobead SX-1 and Dowex AG 1-X8 were prepared by the following wash cycle (10 bed volumes of each): 1 N NaOH; H_2O; 1 N HCl; 1 N HCl (50% ethanol); H_2O. for thiol assay by a quantitative determination of the terminal thiophosphate group, a 0.1-mL column fraction aliquot was diluted to 1 mL with a solution containing 0.1 M phosphate buffer (pH 7.7) and 5 mM DTNB. The absorbance was measured at 412 nm, $\epsilon = 14150\ M^{-1}$.

For the alkaline phosphatase and P_i assay, the 1-mL reaction solution consisted of 0.1 M Ches buffer (pH 9.5), 0.29 mg of alkaline phosphatase, and 50 nmol of phosphate ester. The solution was incubated for ca. 15 min at room temperature. A 0.1-mL reaction mixture aliquot was then assayed for P_i by Lanzetta's et al. (1979) procedure.

^{31}P NMR Spectra. ^{31}P NMR spectra were obtained on a Bruker Instrument Inc. WM-360 spectrometer operating at 145.81 MHz. The characterization spectra were proton decoupled and were measured with 32K data points, a sweep width of 9091 Hz (0.555 Hz/point), a 70° radio-frequency pulse, and an overall repetition rate of 1.802 s. Approximately 2000 scans were acquired for each spectrum. The spectra were resolution-enhanced with a Gaussian window function (GB = 0.020, LB = −1.2). The isotopic shift analysis spectra were measured with 16K data points, a sweep width of 1202 Hz (0.1437 Hz/point), a 85° radio-frequency pulse, and an overall repetition rate of 6.816 s. Approximately 2000 scans were acquired for each spectra. The acquired data were zero filled to 32K data points, and the spectra were resolution enhanced with a Gaussian window function (GB = 0.030, LB = −0.8). The isotopic distribution (%) was calculated by dividing the integrated area of a particular peak by the sum of the integrated areas of all relevant peaks.

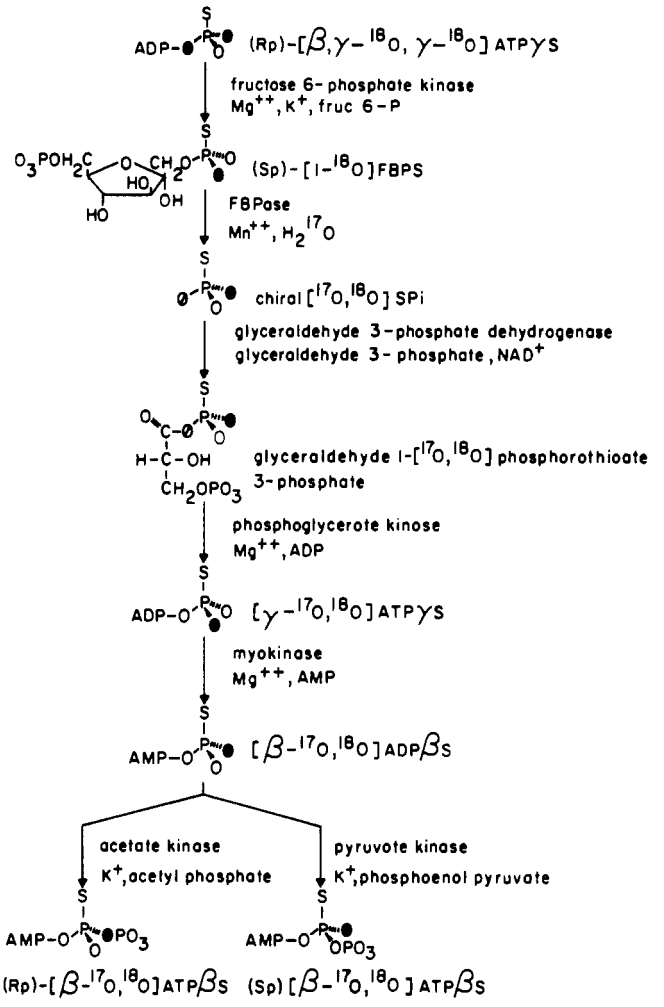
Each sample contained 6–8 mM nucleotide, 20 mM EDTA, 50 mM CHES buffer, pD 10.4, in 2 mL of 99% D_2O (MSD Isotopes). The buffer and D_2O were passed through a Chelex column prior to use. Each NMR sample was then filtered through a 0.22-μm Millex-GV filter (Millipore) into a new NMR tube. The ^{31}P NMR chemical shifts were relative to an external 1.0 M H_3PO_4 standard with downfield being positive.

Gas Chromatography–Mass Spectrometry (GC–MS). A Finnigan 3200 quadrupole GC-MS with a Model 6000 data reduction system and electron-impact ionization was used. Isotopic analysis was performed by selective ion monitoring the effluent of a 2 mm × 1.5 m glass chromatography column packed with 10% Silar 10-C on 100–120 mesh Gas Chrom Q, which had been preconditioned at 200 °C for 3 h with oxygen-free N_2. Triethyl phosphate exhibits a retention time of 8.5 min at 105 °C and 20 mL/min, helium carrier gas flow.

By monitoring the ion intensities in the molecular ion region of m/e 99, 101, 103, 105, and 107, which correspond to the $(HO)_4P^+$ fragment, one can calculate the percentages of unlabeled and higher labeled species. Sharp & Benkovic (1979) have described the protocol for monitoring the intensities of five m/e values.

Synthesis. (A) Synthesis of (S_P)-[1-^{18}O]FBPS. (R_P)-[β,γ-^{18}O,γ-^{18}O]ATPγS was synthesized by the method of Richard & Frey (1978). ^{31}P NMR showed the compound to be 93% ^{18}O enriched in the R_P position. The (S_P)-[1-^{18}O]-FBPS was synthesized enzymatically in a stereospecific reaction catalyzed by fructose-6-phosphate kinase (Jarvest et al., 1981) (Scheme I). The reaction mixture (23.5 mL) contained 43 mM Tris-HCl, pH 8.5, 8.5 mM $MgSO_4$, 25.5 mM KCl, 0.85 mM (R_P)-[β,γ-^{18}O,γ-^{18}O]ATPγS, 1.86 mM DTE, 6.55 mM Fru-6-P, and 55 IU of fructose-6-phosphate kinase. The reaction's progress was followed by TLC system A (R_f of ATPγS 0.15; R_f of FBPS 0.44). After 9 h, the reaction was quenched by the addition of excess EDTA.

Scheme I: Correct Stereochemistry for FBPase-Catalyzed Hydrolysis[a]



[a] The ATPβS species shown represents the major species visible in the ^{31}P NMR spectra. O = ^{16}O; Ø = ^{17}O; ● = ^{18}O.

The reaction mixture was diluted to 50 mL with 0.3 M TEAB, applied to a DEAE-Sephadex A-25 column, and completely resolved with a 4-L linear gradient of 0.3–0.7 M TEAB. The (S_P)-[1-^{18}O]FBPS eluted at 0.41 M TEAB as determined by the thiol assay. Fractions containing the derived compound were pooled, and the solvent was evaporated. The excess buffer was removed by repeatedly dissolving the residue in 95% ethanol and evaporating the solvent. The Büchi rotoevaporator water bath was maintained at 30 °C. This synthesis yielded 16 μmol (80% yield) of (S_P)-[1-^{18}O]FBPS as determined by a coupled alkaline phosphatase–inorganic P_i assay (Lanzetta et al., 1979). ^{31}P NMR showed the β form to be the major anomer (82%) with the α 1-P at 43.9 ppm, the β 1-P at 42.9 ppm, the α 6-P at 1.5 ppm, and the β 6-P at 1.7 ppm. The spectrum indicated that the compound was 94% pure.

(B) Synthesis of [γ-^{17}O,γ-^{18}O]ATPγS. The incorporation of chiral SP_i into ATPγS as described below is a modified version of Webb & Trentham's (1980) procedure and proceeds

with inversion of configuration at phosphorus. Conditions were changed to avoid isotopic washout from the labile intermediate and to increase the overall yield. The dialyzed enzyme mixture contained aldolase (5 IU), glyceraldehyde-phosphate dehydrogenase (1000 IU), lactate dehydrogenase (50 IU), phosphoglycerate kinase (5060 IU), and triosephosphate isomerase (310 IU) with 20 mM Tris-HCl buffer, pH 8.0, 1 mM DTE, and 1 mM EDTA in a total volume of 1.26 mL. To this solution, the following substrates and cofactors were added: 15.5 μmol of chiral SP_i, 100 μmol of ADP, 0.4 μmol of NAD, 80 μmol of $MgCl_2$, 4.5 μmol of DTE, 82 μmol of Fru-1,6-P_2, and 100 μmol of pyruvate in 1 mL of 50 mM Tris-HCl, pH 7.5. The reaction was followed by TLC system A (R_f of ATPγS 0.15; R_f of SP_i 0.20) and was complete after 3 h. The [γ-^{17}O,γ^{18}O]ATPγS was purified as above and eluted at 0.52 M TEAB with 11.6 μmol of ATPγS (73% yield) isolated as determined by UV absorption. The ^{31}P NMR characterization spectrum showed a doublet centered at 32.8 ppm from the γ-P, two doublets centered at −23.5 ppm from the β-P, and a doublet centered at −11.6 ppm from the α-P. The spectrum indicated that the compound was >95% pure.

(C) Synthesis of [β-^{17}O,β-^{18}O]ADPβS. The terminal thiophosphoryl group of ATPγS was stereospecifically transferred to AMP with inversion of configuration at phosphorous by myokinase (Richard & Frey, 1978). The reaction mixture (5 mL) consisted of 50 mM AMP, 25 mM $MgCl_2$, 1 mM DTE, and 100 mM Tris-HCl, pH 8.5, and contained 11.6 μmol of [γ-^{17}O,γ-^{18}O]ATPγS and 2550 IU of dialyzed myokinase. After 2 h, the reaction was complete as determined by TLC system A (R_f of ADPβS 0.28). The [β-^{17}O,β-^{18}O]-ADPβS was purified as above and eluted at 0.43 M TEAB with 11 μmol of ADPβS isolated as determined by UV absorption. The ^{31}P NMR characterization spectrum showed a doublet centered at 31.9 ppm from the β-P and a doublet centered at −12.5 ppm from the α-P and indicated that the compound was >95% pure.

(D) Synthesis of (R_P)-[β-^{17}O,β-^{18}O]ATPβS. [β-^{17}O,β-^{18}O]ADPβS was enzymatically phosphorylated at the *pro-R* position by acetate kinase (Jaffe & Cohn, 1978). The reaction solution (5 mL) consisted of 100 mM Tris-HCl, pH 8.5, 25 mM $MgCl_2$, 100 mM KCl, 7 mM DTE, and 31 mM acetyl phosphate and contained 11.6 μmol of [β-^{17}O,β-^{18}O]ADPβS and 115 IU of dialyzed acetate kinase. Due to the acid lability of ATPβS, the reaction's progress was followed by TLC system B (R_f of ADPβS 0.27; R_f of ATPβS 0.10). After 3 h, the reaction was quenched by the addition of excess EDTA. The ATPβS was purified as above and eluted at 0.53 M TEAB. As determined by UV absorption, a quantitative yield of (R_P)-[β-^{17}O,β-^{18}O]ATPβS was obtained from this conversion with an overall yield from (S_P)-[1-^{18}O]FBPS to (R_P)-[β-^{17}O,β-^{18}O]ATPβS of 70%. The ^{31}P NMR characterization spectrum showed a doublet centered at −6.5 ppm from the γ-P, two doublets centered at 28.8 ppm from the β-P, and a doublet centered at −11.9 ppm from the α-P. The spectrum indicated that the compound was >95% pure.

(E) Synthesis of (S_P)-[β-^{17}O,β-^{18}O]ATPβS. [β-^{17}O,β-^{18}O]ADPβS was enzymatically phosphorylated at the *pro-S* position by pyruvate kinase (Jaffe & Cohn, 1978). The reaction solution (3 mL) consisted of 35 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 75 mM KCl, 1 mM DTE, and 35 mM phosphoenolpyruvate and contained ca. 10 μmol of [β-^{17}O,β-^{18}O]ADPβS and 860 IU of pyruvate kinase. TLC system B was used to follow the reaction's progress. After 6 h the reaction was quenched with excess EDTA. The (S_P)-[β-^{17}O,β-^{18}O]ATPβS was purified as above and eluted at 0.60 M TEAB. The yield of (S_P)-[β-^{17}O,β-^{18}O]ATPβS from this reaction was 8 μmol (80% yield) as determined by UV absorption with an overall yield from (S_P)-[1-^{18}O]FBPS to (S_P)-[β-^{17}O,β-^{18}O]ATPβS of ca. 50%. The ^{31}P NMR characterization spectrum showed a doublet centered at −6.4 ppm from the γ-P, two doublets centered at 29.0 ppm from the β-P, and a doublet centered at −11.9 ppm from the α-P. The spectrum indicated that the compound was >95% pure.

Methods. (A) Hydrolysis of (S_P)-[1-^{18}O]FBPS by FBPase in $H_2{}^{17}O$. Studies of the metal cofactor requirements revealed that the hydrolysis of FBPS was catalyzed by Mn^{2+} only. The FBPase activity is also significantly inhibited by the substrate analogue at 5 mM or greater and by the products SP_i and Fru-6-P at 10 mM or greater.

For the above reasons and in order to limit the amount of $H_2{}^{17}O$ required, the enzymatic hydrolysis was carried out in series. Four aliquots of FBPase, each containing 7.5 mg, were centrifuged, and the enzyme-containing supernatants were lyophilized to dryness. Four tubes containing 3.95 μmol of (S_P)-[1-^{18}O]FBPS, 2 μmol of $MnCl_2$, and 2 μmol of DTE, in 2 mL of 50 mM, Tris-HCl, pH 7.5, were lyophilized to dryness. A sample of the lyophilized FBPase was dissolved in 1 mL of $H_2{}^{17}O$ (12.5% ^{16}O, 52.4% ^{17}O, 35.1% ^{18}O; KOR Inc.). This solution was transferred to one of the tubes containing substrate and cofactors, the latter dissolved, and the reactor's progress (ca. 1 h) was followed by TLC system A (R_f of SP_i 0.20). This procedure was repeated for each of the remaining three tubes after the $H_2{}^{17}O$ had been recovered from the previous reaction by sublimation at 0.01 mm of pressure in a transfer soldier apparatus.

The combined four reaction residues were diluted to 25 mL with H_2O. The chiral SP_i was purified as above and eluted at 0.23 M TEAB. From this hydrolysis, a quantitative yield of chiral SP_i was obtained as determined by the thiol assay.

(B) Hydrolysis of (R_P)-[β-^{17}O,β-^{18}O]ATPβS. The (R_P)-[β-^{17}O,β-^{18}O]ATPβS was hydrolyzed to [β-^{17}O,β-^{18}O]ADPβS with alkaline phosphatase (Newman, 1968). The reaction mixture (5 mL) contained 50 mM CHES buffer, pH 9.5, 2 mM (R_P)-[β-^{17}O,β-^{18}O]ATPβS, and 100 units of alkaline phosphatase. The reaction as followed by TLC system B was complete in 30 min. The (R_P)-[β-^{17}O,β-^{18}O]ADPβS was purified as above. The yield from the dephosphorylation was 10 μmol of (R_P)-[β-^{17}O,β-^{18}O]ADPβS as determined by UV absorption.

(C) Single Turnover in $H_2{}^{18}O$ and with $H_2{}^{18}O$-Equilibrated FBPase. In each of the following experiments, the reaction mixture was incubated at 25 °C for 10 min prior to the addition of the substrate. In the experiments performed in $H_2{}^{18}O$, all components including the substrate and the EDTA quench were lyophilized to dryness and then redissolved in $H_2{}^{18}O$. The enzymic activity was determined by the procedure of Frey et al. (1977) with 124 mM^{-1} as the extinction coefficient at 280 nm. Under the reaction conditions stated below for all the turnover experiments, greater than 95% of the substrate was bound to the FBPase tetramer based on the binding constants determined in the presence of Mg^{2+} (Benkovic et al., 1978).

The single turnover of Fru-1,6-P_2 by FBPase was carried out in 1.0 mL of $H_2{}^{18}O$ (^{16}O, 2.8%; ^{17}O, 1.8%; ^{18}O, 95.4%; Monsanto) that consisted of 80 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA (Benkovic & deMaine, 1982), 100 μM Fru-1,6-P_2, and 400 μM catalytically competent FBPase active sites. The reaction was quenched after 5 s with 0.11 mL of 500 mM EDTA (de Maine & Benkovic, 1980).

The desired products, P_i and FBPase, were separated by passing the undiluted reaction mixture through a 2.5 cm ×

30 cm Sephadex G-25 column that had been preequilibrated with 0.2 M TEAB. The P_i-containing fractions as determined by the procedure of Lanzetta et al. (1979) were pooled and the excess buffer was removed by repeatedly dissolving the residue in 95% ethanol and evaporating the solvent. The Büchi rotoevaporator water bath was maintained at 25 °C. The P_i was dissolved in 5 mL of water and purified from the other byproducts by passage through a 1.5 cm × 8 cm Dowex Ag 1-X8 column employing a 200-mL, 0–40 mM HCl linear gradient. The P_i eluted at 25 mM HCl. The pooled fractions from the Dowex AG 1-X8 column contained, in addition to the P_i, significant amounts of EDTA in the acid form. The EDTA was removed by taking advantage of its insolubility in cold H_2O. The P_i residue was dissolved in 1 mL of cold H_2O, the solution was centrifuged for 2 min at 4000 rpm, and the supernatant was withdrawn. Mass spectral analysis of the P_i at this stage of purification showed the sample to be contaminated with plasticizers that interfered with the GC–MS analysis. Those contaminants were removed by diluting the P_i sample to 4 mL with water, passing the solution through a 0.5 cm × 4 cm Biobead column, and washing the column with 2 bed volumes of water. The plasticizers were retained, leaving the P_i-containing eluant free of all contaminants. This eluant was lyophilized to dryness, dissolved in 50 μL of 95% ethanol, and stored at −75 °C.

The enzyme-containing fractions from the Sephadex G-25 column were pooled and dialyzed against saturated ammonium sulfate, pH 7.5 at 5 °C. The enzyme was precipitated by centrifugation at 20000g. The ammonium sulfate was removed by extensive dialysis against 10 mM Tris-HCl, pH 7.5 at 5 °C, which left the enzyme sample ready for the next experiment.

The above FBPase was ^{18}O equilibrated through multiple turnovers in $H_2{}^{18}O$. The 1.0-mL reaction mixture contained 80 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 110 μM Fru-1,6-P_2, and 220 μM catalytically competent FBPase active sites. The reaction was quenched after 1 min with 0.11 mL of 500 mM EDTA. The P_i and $H_2{}^{18}O$-equilibrated FBPase were separated and purified as above.

The single turnover of Fru-1,6-P_2 by $H_2{}^{18}O$-equilibrated FBPase in 6 mL of $H_2{}^{16}O$ contained 80 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 44 μM Fru-1,6-P_2, and 88 μM catalytically competant FBPase active sites. The reaction was quenched after 5 s with 0.67 mL of 500 mM EDTA. The P_i and enzyme were separated and purified as above.

The P_i was ethyl esterified by diazoethane. The diazoethane was cautiously prepared just prior to use from *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine by dissolving 1 g of this precursor in a cold, stirring solution of 3 mL of 40% KOH and 15 mL of dry, diethyl ether in a 250-mL Erlenmyer flask. The diazoethane ethereal solution was distilled into a 25-mL screw-capped, cold ether containing test tube. The final solution was dark orange in color. Each ethanolic P_i sample was diluted with 2 mL of the diazoethane solution and incubated for 5 min at 23 °C. The samples were then concentrated to 15 μL with a stream of dry N_2(g) at 20 °C.

Results and Discussion

The hydrolysis of (S_P)-[1-^{18}O]FBPS *in* $H_2{}^{17}O$ catalyzed by FBPase generates chiral [^{17}O,^{18}O]SP_i in either of two configurations, (R_P)-[^{17}O,^{18}O]SP_i via retention or (S_P)-[^{17}O,^{18}O]SP_i via inversion, or as a racemate. To determine the configuration of the chiral SP_i, the compound was enzymatically incorporated into [β-^{17}O,β-^{18}O]ATPβS of known configuration with enzymes whose stereochemical course of phosphoryl transfer was established (Webb & Trentham, 1980; Richard & Frey, 1978) (Scheme I).

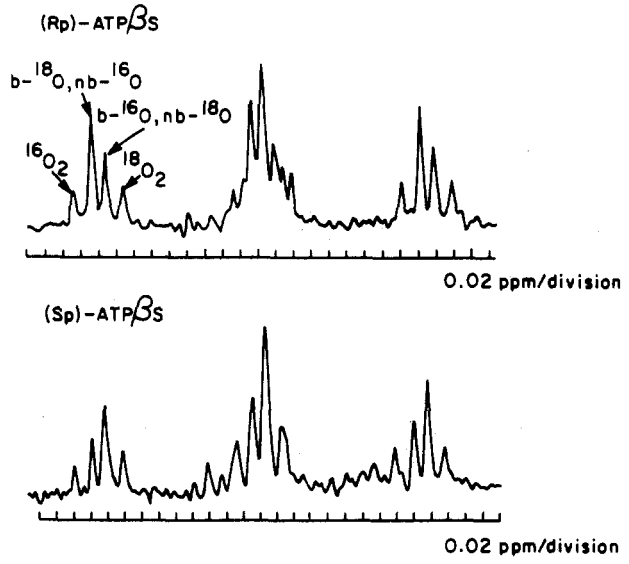


FIGURE 1: β-Phosphorus region of (R_P)- and (S_P)-[β-^{17}O,β-^{18}O]-ATPβS. See text for details. The ATPβS species are designated by their oxygen isotopes in the β,γ-bridging (b) and the β-nonbridging (nb) positions.

To verify the position of the isotopic enrichment, the [β-^{17}O,β-^{18}O]ADPβS was first phosphorylated at the *pro-R* position forming (R_P)-[β-^{17}O,β-^{18}O]ATPβS with acetate kinase and then analyzed by ^{31}P NMR. The (R_P)-[β-^{17}O,β-^{18}O]-ATPβS was subsequently dephosphorylated to [β-^{17}O,β-^{18}O]ADPβS, then phosphorylated at the *pro-S* position forming (S_P)-[β-^{17}O,β-^{18}O]ATPβS with pyruvate kinase, and again analyzed by ^{31}P NMR. The stereochemical analysis of [β-^{17}O,β-^{18}O]ATPβS exploits ^{18}O-induced upfield chemical shifts (Cohn & Hu, 1978; Lowe et al., 1979) and ^{17}O-induced line broadening (Tsai, 1979) of the ^{31}P NMR signal to establish the labeling pattern for the chiral [^{17}O,^{18}O]SP_i. The ^{31}P NMR isotopic analysis spectra for both [β-^{17}O,β-^{18}O]ATPβS samples are exhibited in Figure 1.

During the reaction catalyzed by phosphoglycerate kinase, the chiral SP_i losses one of its oxygens (Webb & Trentham, 1980). The ultimate ATPβS species derived from [β-^{17}O,β-^{18}O]ATPβS that has lost ^{17}O ([β-^{18}O]ATPβS) will be the most intense peak in the ^{31}P NMR spectrum and will have one ^{18}O bound to the β-P. The magnitude of the upfield chemical shift for this species depends on whether the ^{18}O is in the bridging position (0.021 ppm) or the nonbridging position (0.037 ppm). The ^{31}P NMR isotopic analysis spectra are complicated by the ATPβS species derived from partial isotopic enrichment since the $H_2{}^{17}O$ consists of all three oxygen isotopes and the (R_P)-[β,γ-^{18}O,γ-^{18}O]ATPγS used to synthesize the (S_P)-[1-^{18}O]FBPS was ca. 93% ^{18}O enriched. Table I shows the predicted distribution for the two possible stereochemical outcomes and the observed distribution.

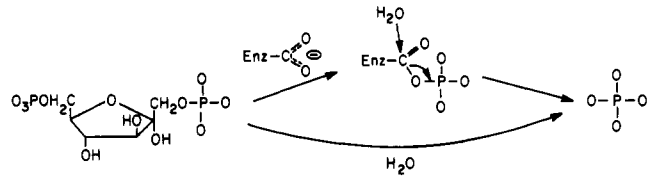
The ^{31}P NMR spectral results are conclusive. The upfield chemical shifts of the most intense peaks in the (R_P)- and (S_P)-[β-^{17}O,β-^{18}O]ATPβS spectra due to the [β-^{18}O]ATPβS species indicate that the ^{18}O isotopic enrichment was located solely in the *pro-R* position. This outcome mandates that the (S_P)-[1-^{18}O]FBPS was hydrolyzed by FBPase with inversion of configuration at the phophoryl center. The simplest mechanistic explanation that satisfies the above result is that the thiophosphoryl group was transferred directly from the substrate analogue to water by an in-line displacement mechanism.

Table I: Isotopic Distribution in the β-Phosphorus of [β-^{17}O,β-^{18}O]ATPβS

species[a]	predicted values (%) retention	predicted values (%) inversion	obsd values (%)
(R_P)-[β-^{17}O,β-^{18}O]ATPβS			
$^{16}O_2$	10	10	15
b-^{18}O, nb-^{16}O	24	49	41
b-^{16}O, nb-^{18}O	49	24	28
$^{18}O_2$	17	17	16
(S_P)-[β-^{17}O,β-^{18}O]ATPβS			
$^{16}O_2$	10	10	13
b-^{18}O, nb-^{16}O	49	24	26
b-^{16}O, nb-^{18}O	24	49	42
$^{18}O_2$	17	17	20

[a] The ATPβS species are designated by their oxygen isotopes in the β,γ-bridging (b) and the β-nonbridging (nb) positions.

Scheme II[a]



[a] Enz-CO_2^- represents FBPase with the active site carboxyl group.

FBPase along with alkaline phosphatase (Jones et al., 1978), acid phosphatase (Saini et al., 1981), and glucose 6-phosphatase (Lowe et al., 1982) hydrolyzes sugar phosphates and phosphate monoesters, yet the stereochemical course of the hydrolysis catalyzed by these latter phosphohydrolases proceeds with retention of configuration. These results suggest E–P intermediates, and indeed, in each case an amino acid residue has been implicated [Coleman & Chlebowski (1979), Van Etten & Hickey (1977), and Feldman & Butler (1972), respectively]. Therefore, our result dictates that there are apparently two stereochemically distinct mechanisms for the hydrolysis of sugar phosphates.

Chemical modification studies with rabbit liver FBPase (J. F. Rahil, M. M. deMaine, and S. J. Benkovic, unpublished results) indicate that a carboxyl group may be essential for activity. Thus, it is conceivable that FBPase-catalyzed hydrolysis includes a covalently bound E–P intermediate like other phosphohydrolases yet follows a mechanism that leads to inversion at the phosphorous center. In this mechanism a carboxyl group forms an acyl phosphate intermediate during hydrolysis, which, in turn, is hydrolyzed by attack at the carboxyl carbon (Scheme II). Given that the stereochemical course entails a two-step reaction sequence involving inversion followed by retention of configuration at phosphorus, the overall result would be inversion of configuration. This type of mechanism has been considered for staphylococcal nuclease (Mehdi & Gerlt, 1982) and eliminated in the case of yeast inorganic pyrophosphatase (Gonzalez et al., 1984).

In order to distinguish between a simple in-line transfer process and one containing an enzyme–acyl phosphate intermediate, two experiments were carried out. A putative carboxyl function was isotopically equilibrated with medium $H_2{}^{18}O$ through multiple turnover of the enzyme. A subsequent single turnover of this ^{18}O-equilibrated enzyme in water should then yield an equivalent of [^{18}O]P_i. Moreover, the presence of covalent carboxyl group participation during FBPase turnover would produce with native FBPase unlabeled P_i after a single turnover in $H_2{}^{18}O$. In the absence of an enzyme–acyl phosphate intermediate, the two experiments would produce unlabeled and ^{18}O-labeled P_i, respectively.

Table II: Predicted P_i Distribution for Two Hydrolytic Processes

single turnover expt with	process	m/e[a]
^{18}O-equilibrated FBPase in $H_2{}^{16}O$	with E–P[b]	101
	without E–P	99
native FBPase in $H_2{}^{18}O$	with E–P	99
	without E–P	101

[a] This ion accounts for ca. 98% of distribution prior to $P_i \rightleftharpoons H_2{}^{18}O$ equilibration process. [b] Enzyme–acyl phosphate intermediate.

Table III: Percent P_i Distribution for Single Turnover with ^{18}O-Equilibrated FBPase in H_2O

m/e	predicted values with E–P[b]	predicted values without E–P	obsd[a]
99	4	98	91
101	80	1	2
103	9	0	1
105	5	0	3
107	2	1	3

[a] The $P^{16}O_4$ contamination has been subtracted. [b] Enzyme–acyl phosphate intermediate.

Table IV: Percent P_i Distribution for Single Turnover with Native FBPase in $H_2{}^{18}O$

m/e	predicted values with E–P[b]	predicted values without E–P	obsd[a]
99	67	3	20
101	17	58	43
103	9	19	20
105	4	13	11
107	1	7	4

[a] The $P^{16}O_4$ contamination has been subtracted. [b] Enzyme–acyl phosphate intermediate.

The minimum time requirement for complete labeling of a putative carboxyl group by medium $H_2{}^{18}O$ under reversible conditions was readily calculated from the turnover number of the enzyme statistically corrected for the two exchangeable oxygens per carboxyl group and the four active sites per molecule. Fully labeled FBPase is achieved in 20 s, an actual incubation time of 1 min was used. Because the P_i product in the presence of Fru-6-P can undergo enzyme-catalyzed equilibration with medium $H_2{}^{18}O$ owing to the resynthesis of Fru-1,6-P_2, the single-turnover experiments were quenched at 5 s to minimize this $P_i \rightleftharpoons {}^{18}O$ equilibration. For each of the two experiments and each of the two mechanisms the initially formed P_i is ca. 98% of one ion (see Table II and below for discription of fragmentation pattern). The amount of $P_i \rightleftharpoons H_2{}^{18}O$ equilibration after 5 s was calculated from the data and Scheme II of Sharp & Benkovic (1979) where $k_{on} = 2.3$ mM^{-1} s^{-1} and $k_{ex}/k_{off} = 1.4$. This equilibration redistributes the major ion as shown under predicted values in Tables III and IV.

Inorganic phosphate from the above series of turnover experiments was isolated, ethylated, and analyzed by GC-MS selective ion monitoring for isotopic enrichment. At 70 eV, the base peak in the fragmentation of triethyl phosphate is $(HO)_4P^+$ of m/e 99. This is an ideal ion for isotopic enrichment experiments since all oxygens of the parent ion remain and this region is void of other fragments.

Control experiments had shown that some contamination by extraneous P_i is unavoidable in view of the small amount of P_i being isolated: ca. 200 nmol. Results from several trial runs under our experimental conditions indicate that the contamination remained fairly constant. The experiments equilibrating the enzyme with medium $H_2{}^{18}O$ through multiple

turnovers provided an estimate of the percent contamination. Under this condition the product P_i will be at least singly labeled owing to $P_i \rightleftharpoons H_2{}^{18}O$ exchange. Because adventitious P_i introduced subsequently would be unlabeled, it would yield an m/e 99 ion in the GC–MS analysis. A comparison of the relative intensity of the m/e 99 ion to the sum of the intensities of the ^{18}O-labeled ions (m/e 99 + 2n, when n = 1–4) indicated that the contaminant P_i and the hydrolytic P_i are both 150–250 nmol. This contribution to the m/e 99 ion was subtracted from all the turnover experimental results. In addition, the P_i formed during the ^{18}O equilibration experiments was distributed mainly between the m/e 105 and m/e 107 ion peaks, confirming that the enzyme had the opportunity to become fully labeled. The observed distribution of ^{18}O-labeled P_i species was in good agreement with that predicted by the kinetic data of Sharp & Benkovic (1979).

Tables III and IV list the distribution of P_i species expected for the two possible mechanisms (with E–P or without E–P) and the results of the two single turnover experiments. Owing to the magnitude of contamination, there is a 20% uncertainty in this observed distribution, but the difference in predicted distribution for the two mechanisms is large enough to more than compensate for the contamination-induced error. The results in Table III indicate that the P_i was not labeled; thus, the ^{18}O equilibration experiment did not label a carboxyl group that participated in the hydrolysis of Fru-1,6-P_2. The results in Table IV indicate that 80% of the P_i was labeled in a single turnover. Collectively, these data mandate the absence of an enzyme–acyl phosphate intermediate during FBPase-catalyzed hydrolysis of Fru-1,6-P_2.

The results from the stereochemical experiment in conjunction with the single-turnover experiments unambiguously demonstrate that the substrate Fru-1,6-P_2 is hydrolyzed without covalent participation of the enzyme. Consequently, phosphohydrolase enzymes catalyze the hydrolysis of sugar phosphates and phosphate monoesters by two mechanisms, through an E–P intermediate as exemplified by alkaline phosphatase, acid phosphatase, and glucose 6-phosphatase and through a single in-line transfer as exemplified here by rabbit liver FBPase.

ADDED IN PROOF

The predicted and observed values in Table I can be brought into exact agreement by adjusting the predicted values for the isotopic dilution that occurs during the enzymatic analysis of SP_i.

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REFERENCES

Benkovic, S. J., & de Maine, M. M. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 45–82.

Benkovic, P. A., Frey, W. A., & Benkovic, S. J. (1978) *Arch. Biochem. Biophys. 191*, 719–726.

Benkovic, P. A., Hegazi, M., Cunningham, B. A., & Benkovic, S. J. (1979) *Biochemistry 18*, 830–835.

Cohn, M., & Hu, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 200–203.

Coleman, J. E., & Chlebowski, J. F. (1979) *Adv. Inorg. Biochem. 1*, 1–66.

de Maine, M. M., & Benkovic, S. J. (1980) *Arch. Biochem. Biophys. 205*, 308–314.

Feldman, F., & Butler, L. G. (1972) *Biochim. Biophys. Acta 268*, 698–710.

Frey, W. A., Fishbein, R., deMaine, M. M., & Benkovic, S. J. (1977) *Biochemistry 16*, 2479–2484.

Gonzalez, M. A., Webb, M. R., Welsh, K. M., & Cooperman, B. S. (1984) *Biochemistry 23*, 797–801.

Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652–657.

Jarvest, R. L., Lowe, G., & Potter, B. V. L. (1981) *Biochem. J. 199*, 427–432.

Jones, S. R., Kindman, L. A., & Knowles, J. R. (1978) *Nature (London) 275*, 564–565.

Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95–97.

Lowe, G., & Potter, B. V. L. (1982) *Biochem. J. 201*, 665–668.

Lowe, G., Potter, B. V. L., Sprout, B. S., & Hull, W. E. (1979) *J. Chem. Soc., Chem. Commun.*, 733–735.

Mehdi, S., & Gerlt, J. A. (1982) *J. Am. Chem. Soc. 104*, 3223–3225.

Newman, H. (1968) *J. Biol. Chem. 243*, 4671–4676.

Pontremoli, S., Traniello, S., Luppis, B., & Wood, W. A. (1965) *J. Biol. Chem. 240*, 3459–3463.

Rahil, J. F., de Maine, M. M., & Benkovic, S. J. (1982) *Biochemistry 21*, 3358–3363.

Richard, J. P., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7757–7758.

Richard, J. P., Ho, H.-T., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7756–7757.

Saini, M. S., Buchwald, S. L., Van Etten, R. L., & Knowles, J. R. (1981) *J. Biol. Chem. 256*, 10453–10455.

Sharp, T. R., & Benkovic, S. J. (1979) *Biochemistry 18*, 2910–2916.

Tsai, M.-D. (1979) *Biochemistry 18*, 1468–1472.

Van Etten, R. L., & Hickey, M. E. (1977) *Arch. Biochem. Biophys. 183*, 250–259.

Webb, M. R., & Trentham, D. R. (1980) *J. Biol. Chem. 255*, 1775–1779.