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Fructose 1,6-Bisphosphatase from Rabbit Liver. [^{18}O]Phosphate–H_2O Exchange as a Probe of the Catalytic Mechanism†

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ABSTRACT: Fructose 1,6-bisphosphatase (FBPase, EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase) has been found to catalyze the solvent exchange of ^{18}O from initially highly enriched inorganic phosphate (P_i). The exchange occurs with either Mg^{2+} or Mn^{2+} as the cofactor. The exchange proceeds negligibly in the presence of P_i alone but is greatly stimulated by the presence of fructose 6-phosphate, the second product of the FBPase reaction. A theoretical treatment of ^{18}O exchange from P_i is presented, based upon the ability to determine the ^{18}O isotopic distribution in a P_i sample. The analytical technique permits the determination of the percentage of P_i molecules in a sample which contain none, one, two, three, or four ^{18}O atoms per P_i molecule, in addition to the total atom percent ^{18}O of the sample. Comparative data are presented, illustrating the sensitivities and precision of an ^{18}O-shifted ^{31}P NMR technique and the mass spectrometric method, in which the latter is shown to be approximately 10^2–10^3 times more sensitive and considerably more precise in the determination of low abundance species. The theoretical treatment defines the conditions under which more than one ^{18}O atom can be exchanged per interaction of a P_i molecule with an enzyme. The quantitative features of exchange time courses, expressed in terms of a rate of exchange/rate of dissociation partition coefficient (k_x/k_{off}), are described. Dissociation of P_i from either Mn^{2+}– or Mg^{2+}–FBPase under equilibrium isotope exchange conditions is sufficiently slow so that exchange of more than one ^{18}O can occur per protein–ligand interaction: k_x/k_{off} = 1.4–2.0. However, during steady-state hydrolysis of fructose 1,6-bisphosphate in [^{18}O]H_2O, only one oxygen of the P_i produced becomes equilibrated with the solvent, predicting a $k_x/k_{off} \leq 0.01$. Such results suggest that either (1) the intermediates involved in equilibrium exchange are different than those involved in hydrolysis or (2) the state of occupancy of the active sites in the enzyme tetramer may control the apparent dissociation rates. However, the observed equilibrium exchange suggests a possible net reversal by FBPase of the hydrolysis reaction.

Fructose 1,6-bisphosphatase (FBPase,[1] EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase) catalyzes the hydrolysis of fructose 1,6-bisphosphate (fru-1,6-P_2) to fructose 6-phosphate (fru-6-P) and inorganic phosphate (P_i). Phosphorus–oxygen bond cleavage occurs during hydrolysis, as inferred from information available from other phosphohydrolases and from ^{18}O incorporation experiments in which hydrolysis of fru-1,6-P_2 is carried out in [^{18}O]H_2O (Pontremoli et al., 1965).

Two points concerning this catalysis have not been established to date: (1) whether fru-1,6-P_2 hydrolysis by FBPase proceeds through a phosphoryl enzyme intermediate and (2) whether the reaction is reversible—i.e., if fru-1,6-P_2 can be formed from fru-6-P and P_i. We have addressed experiments toward testing these two points by utilizing oxygen isotope exchange reactions catalyzed by FBPase. Analysis for ^{18}O

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[1] Abbreviations used: FBPase, fructose 1,6-bisphosphatase; fru-1,6-P_2, fructose 1,6-bisphosphate; fru-6-P, fructose 6-phosphate; P_i, inorganic phosphate; P_n, inorganic labeled with ^{18}O, n denoting the number of ^{18}O atoms per phosphate molecule, e.g., P_3 contains three ^{18}O atoms per phosphate molecule; NMR, nuclear magnetic resonance; GC–MS, gas chromatograph–mass spectrometer.

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distribution as well as content has required us to develop a theoretical framework within which the data obtained here, as well as data from experiments with other systems, can be analyzed. Relationships between the rate constants that partition P_i between dissociation from an enzyme–phosphate complex and oxygen exchange can be determined under certain experimental conditions.

Experiment Procedures

Materials. FBPase was prepared from rabbit liver by the method of Ulm et al. (1975) as modified by Benkovic et al. (1974). Enzymic activity was assayed by the Mg^{2+}–EDTA assay of Frey et al. (1977), in which the absorbance change at 340 nm due to production of NADPH is followed by employing hexokinase and glucose-6-phosphate dehydrogenase as coupling enzymes. FBPase concentrations were measured by using an extinction coefficient at 280 nm of 0.720 for a 1 mg/mL solution.

[^{18}O]H_2O was obtained from Bio-Rad Laboratories (10% enrichment) or Miles Laboratories (98% enrichment). Fru-1,6-P_2, fru-6-P, glucose-6-phosphate isomerase, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. The fru-6-P analogue (α + β)-methyl D-fructofuranoside 6-phosphate was a gift of Dr. R. Fishbein (Fishbein et al., 1974). The Tris base used in buffers was first recrystallized from 95% ethanol made 0.01% with EDTA in order to eliminate trace metals.

Exchange Reactions. Exchange reactions were routinely carried out in 50 mL of solution containing 50 mM Tris-HCl buffer, pH 7.5, at 25 °C. Reactions with Mg^{2+} and EDTA were 5 mM $MgCl_2$ and 0.1 mM EDTA. Reactions with Mn^{2+} contained 0.1 mM $MnCl_2$. Aliquots of [^{18}O]phosphate and fru-6-P stock solutions were added to reaction mixtures to make them 0.20 mM in each component, when present. Variances in the above conditions will be noted for a given experiment.

Fru-6-P concentrations were determined by using a modification of the FBPase activity assay of Frey et al. (1977). P_i determinations were performed by using the method of Ames (1966). The Mg^{2+}–EDTA/Mn^{2+} activity ratios for the different enzyme preparations used in this study were measured as in Frey et al. (1977) and were found for one preparation to be 2.0 and for another to be 1.6, in comparison with a ratio of 2.75 reported by Frey et al. (1977).

[^{18}O]Phosphate Analysis. [^{18}O]Phosphate was prepared by equilibrating KH_2PO_4 with [^{18}O]H_2O at 110 °C for an extended time, according to the method of Boyer & Bryan (1967).

^{18}O analysis of P_i was accomplished essentially by the method of Midelfort & Rose (1976). The P_i was isolated from reaction mixtures by ion-exchange chromatography on 1 × 4 cm columns of Dowex AG 1-X8 Cl^-, 100–200 mesh (Bio-Rad Laboratories), equilibrated with distilled water. Samples, in which the enzymic reaction had been stopped by addition of KOH, were neutralized and applied to the columns. The columns were washed with distilled water. The P_i was eluted from the columns with 10 mM HCl. Fractions containing P_i were pooled and lyophilized.

After lyophilization, the phosphoric acid residue was methyl-esterified with an excess of an ethereal solution of diazomethane, after dissolving the residue in approximately 1 mL of 10% H_2O in methanol. Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Co.) according to the method of Fieser & Fieser (1967). The samples, after concentration to a small volume, were analyzed for ^{18}O content by gas chromatography–mass spectrometry–selected ion monitoring.

Table I: Determination of ^{18}O Content of Inorganic Phosphates Comparison of GC-MS-SIM and ^{18}O-Shifted ^{31}P NMR Methods

	measured by ^{31}P NMR[b] (%)		measured by GC-MS-SIM[c] (%)	
labeled[a] species	obsd[d]	expected[e]	obsd[f]	expected[c]
P_0	1.0	0.2	0.4	0.2
P_1	2.6	3.1	3.2	3.3
P_2	16.2	17.1	17.3	17.6
P_3	41.6	41.6	41.9	41.7
P_4	38.6	38.0	37.2	37.2
atom % ^{18}O[g]	78.6		78.1	

[a] Subscripts refer to the number of ^{18}O atoms contained in a given P_i molecule. [b] Required ~10–30 μmol of P_i. [c] Required ~10–30 nmol of P_i. [d] Spectrum was integrated by cutting out and weighing peaks from a photocopy. [e] Calculated from the observed atom percent ^{18}O by expansion of the binomial $(a + b)^4$. [f] Data from GC-MS-SIM measurements of trimethyl phosphate. [g] Calculated from the observed data according to the method of Eargle et al. (1977).

A Finnigan 3200 quadrupole gas chromatograph–mass spectrometer (GC–MS) with a Model 6000 data reduction system and electron impact ionization was used.[2] Isotopic analysis was performed by selected ion monitoring of the effluent of a 2 mm i.d. × 1.5 m glass chromatography column packed with 10% Silar 10-C on 100–120 mesh Gas-Chrom Q (Applied Sciences Laboratories). Trimethyl phosphate exhibits a retention time of approximately 6 min at 140 °C and 20 mL/min helium carrier gas flow.

The electron impact mass spectrum of trimethyl phosphate observed here agrees well with that reported by Bafus et al. (1966). Monitoring of the ion intensities in the molecular ion region, at *m/e* 140, 142, 144, 146, and 148, allows calculations of the total ^{18}O content of a trimethyl phosphate sample, as well as the percentages of unlabeled and singly, doubly, triply, and quadrupally labeled trimethyl phosphate in the sample. Because the Model 6000 data system is constructed to monitor intensities at a maximum of four *m/e* settings, the following protocol has been adopted in order to obtain intensities at the five desired *m/e* values.

A series of five injections of a given sample are made into the GC–MS. For each injection, one of the five combinations of the five *m/e* values taken four at a time is observed. The intensities observed from these five injections are compared, and the missing intensity of each set of numbers is estimated.[3] The percentage of the total for the various labeled species and the total ^{18}O content of the trimethyl phosphate sample is then calculated as described by Eargle et al. (1977).

The highly enriched [^{18}O]phosphate prepared for these experiments has been analyzed by the mass spectrometric method described above and by a recently described NMR technique (Cohn & Hu, 1978).[4] Excellent agreement on this material was obtained by the two methods, as is shown in Table I. In addition, calculation of the expected percentages of each of the isotopomers for a random distribution of given atom percent ^{18}O agrees with those found.[5] The noteworthy

[2] Mass spectrometry facilities, Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

[3] The algorithm for estimation of the missing intensities has been programmed in Fortran. Readers desiring information should contact the authors.

[4] Performed by Dr. M. Balakrishnan on the 360-MHz NMR at the Middle Atlantic NMR Facility, University of Pennsylvania, Philadelphia, PA.

[5] Calculated by evaluation of the five terms of the expanded expression $(a + b)^4$, where a = atom fraction of ^{16}O and b = atom fraction of ^{18}O.

differences between analysis by the two methods are as follows. First, the limit for precise measurement of low-intensity species is ~5% for the NMR method, due to signal to noise considerations. For the mass spectral method, the limit is 0.5%. Second, the NMR measurement requires a total of 10–20 μmol of [^{18}O]P_i in ~1.5 mL, whereas the minimal amount of trimethyl [^{18}O]phosphate for the mass spectral method is 10–50 nmol. We have routinely obtained measurements by this GC–MS method with precisions of ±1%.

^{18}O Incorporation during Hydrolysis of Fru-1,6-P_2. Experiments in which fru-1,6-P_2 was hydrolyzed by FBPase in [^{18}O]H_2O were carried out. The following components were placed in a 4-mL cuvette: 200 μmol of Tris-HCl, pH 7.5, 50 μmol of NADP, 30 units of glucose-6-phosphate isomerase, 7 units of glucose-6-phosphate dehydrogenase, 0.05 unit of FBPase, and either 20 μmol of $MgCl_2$ and 1 μmol of EDTA or 0.4 μmol $MnCl_2$. [^{18}O]H_2O was added to make approximately 4 mL of total volume. After incubation at 25 °C for 10 min, fru-1,6-P_2 was added and the ΔA_{340} observed. For the 100% hydrolysis determination, 2 μmol of fru-1,6-P_i was added and the reaction allowed to proceed to completion. For the low-percentage conversion determinations, 20 μmol of fru-1,6-P_2 was added, and the reaction was stopped at approximately 10% hydrolysis by vortexing the reaction mixture with 0.5 mL of $CHCl_3$. The [^{18}O]H_2O was recovered for later use by lyophilization. The residues were dissolved in H_2O and chromatographed as described above to isolate the P_i for analysis.

Analysis for ^{18}O Content of H_2O. The ^{18}O content of H_2O for hydrolysis experiments carried out in [^{18}O]H_2O was measured by hydrolysis of a few granules of PCl_5 with a 10-μL aliquot of the final reaction solution. The resulting H_3PO_4 was methyl-esterified and analyzed for ^{18}O content as described above.

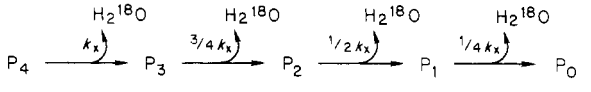
Reaction Simulations. Simulations of the theoretical models considered here, as well as fittings of models to experimental data, were performed by using an interactive chemical reaction simulator program written in Fortran for the departmental computer system by Dr. William E. Brugger. The algorithm used (Shindell & Magagnosc, 1976) calculates the reaction time course by means of Taylor series expansions of the differential equations that describe the changes in concentration of the chemical species in the system.

Theory

The multiplicity with which P_i can be isotopically labeled by ^{18}O makes it necessary to incorporate this feature into the construction of a model for a reaction system. Methods prior to the development of the GC–MS method used here [Midelfort & Rose (1976); see also Bar-Tana et al. (1972)] gave only the total ^{18}O content of a given P_i sample [e.g., Anbar et al. (1960), Boyer et al. (1961), Boyer & Bryan (1967), and Cohn (1957)]. Information regarding the distribution of ^{18}O in a P_i sample could simply not be obtained. The additional information provided by this analytical technique necessitates a systematic development of the theory of this type of exchange system. Such development will be set forth here, by expanding upon the preliminary discussions of Eargle et al. (1977).

Simple Statistical Model. The model described by Eargle et al. (1977) is shown here as Scheme I. The model assumes

Scheme I



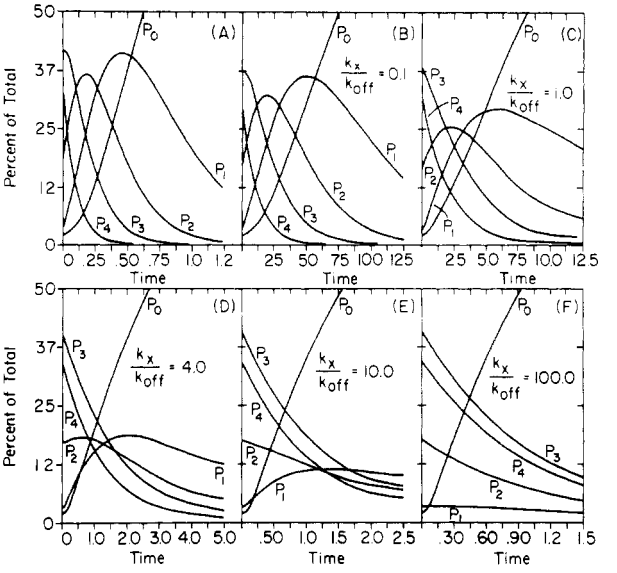
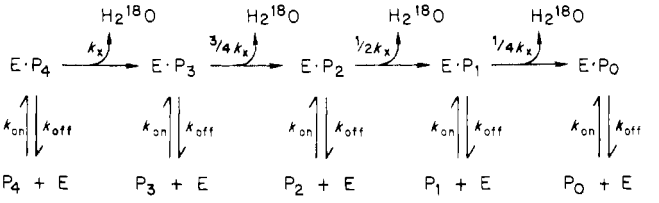


FIGURE 1: Simulations of the [^{18}O]phosphate exchange models depicted in Schemes I and II. The following are the initial percents of the total for all simulations: P_4 = 35%; P_3 = 41.5%; P_2 = 18%; P_1 = 3.5%; P_0 = 2%. (A) Scheme I, with k_x = 10. (B–F) Scheme II, with k_{on} = 1.0, k_{off} = 10.0. (B) k_x = 1.0; (C) k_x = 10.0; (D) k_x = 40.0; (E) k_x = 100.0; (F) k_x = 1000.0.

Scheme II



the chemical equivalence of all four oxygens of P_i and that, if binding of a phosphate group involves interaction through one or more phosphate oxygens, the rate of interchange of bound and nonbound oxygen(s) is fast with respect to any other chemical event. The rate of ^{18}O loss is described by $k_x[P_n]S$, where S is a statistical factor expressing the probability that the oxygen atom to be lost in an exchange process will be an ^{18}O atom. The exchange reaction is indicated as being irreversible because $H_2{}^{18}O$ released from the exchange reaction is assumed to be free to equilibrate with the bulk solvent, where it is diluted to a negligible concentration.

A simulation of this model is included here for comparison with subsequent simulations (Figure 1A). The fit of experimental data obtained by Eargle et al. (1977) to this model for alkaline phosphatase catalyzed exchange of the oxygen of P_i with water at pH 7.0 is excellent. Similar P_i–H_2O exchange data obtained by Van Etten & Risley (1978) for human prostatic acid phosphatase and by Balakrishnan et al. (1978) for glutamine synthetase also fit this model well.

Enzyme-Bound Exchange Model. Scheme II shows an expanded model incorporating the binding of P_i to a protein, in addition to the features of the simple statistical model described above. The following points will be made in contrasting the two schemes.

With the simple statistical model, only one ^{18}O can be lost per interaction of protein and P_i. With the enzyme-bound model, it is possible to exchange more than one ^{18}O per interaction of protein and P_i. That data for an enzymic system would fit the simple statistical model implies that the release of P_i into solution is fast with respect to the exchange step—i.e.,

$k_{off} >> k_x$ (Wimmer & Rose, 1978). Indeed, if a simulation of the model of Scheme II is performed such that $k_x/k_{off} = 0.1$, the qualitative appearance of the time courses for ^{18}O exchange from either model becomes indistinguishable (compare Figure 1B with 1A).

As the value of k_x becomes comparable to and exceeds that of k_{off}, the behavior of the model in Scheme II changes dramatically. A series of simulations, in which k_x/k_{off} is varied from 0.1 to 100, is shown in Figure 1B–F. Several qualitative features of the time courses can be seen to change across this series. With $k_x/k_{off} = 0.1$, concentrations of the species P_3, P_2, and P_1 rise to maxima and then decay (Figure 1B). As k_x/k_{off} increases to and exceeds 1.0, the maximum for P_3 disappears and the relative heights of the maxima for P_2 and P_1 change such that, at $k_x/k_{off} = 4.5$, they are approximately equal (Figure 1C–E). At $k_x/k_{off} = 10.0$, the maximum for P_2 has all but disappeared (Figure 1E). At $k_x/k_{off} = 50.0$, P_1 remains at a constant low level, and at $k_x/k_{off} = 100.0$ (Figure 1F), P_1 decreases continuously. Concurrently, the relationship of the initial rates of increase of P_1 and P_0 changes. As k_x/k_{off} increases to and exceeds 1.0, the rates of increase in P_1 and P_0 become closer, and by $k_x/k_{off} = 4.0$, the rate of increase of P_0 exceeds that of P_1 (Figure 1C–F).

Kinetic Distinguishability. If the time courses for the change in atom percent ^{18}O are plotted for the simulations shown in Figure 1, pseudo-first-order curves are obtained. This feature points out a powerful capability of the type of treatment described here. All the cases presented here are kinetically indistinguishable if the exchange of ^{18}O is followed by measurement of atom percent ^{18}O. However, when distributional analysis is used as the analytical technique, the above cases can be readily distinguished. In terms of Scheme II the value of the first-order rate constant for the exchange from the P_4 species is given by $k'_{on}k_x/(k_{off} + k_x)$.

Results

[^{18}O]Phosphate Exchange. Figure 2 shows a time course for the exchange of ^{18}O from P_i in the presence of 0.5 mM Mn^{2+}, 0.2 mM [^{18}O]P_i (initially 78 atom % ^{18}O), and 0.2 mM fru-6-P. The enzyme concentration was determined to be 1.1 mM in active sites, based upon a monomer weight of 35 000. The metal ion concentration is the optimal concentration for enzyme activity, as indicated by previous work (Frey et al., 1977; Dudman et al., 1978). Concentrations of P_i and fru-6-P were chosen based upon binding studies (Benkovic et al., 1978) so that at least two active sites per tetramer would be saturated with each ligand. Experiments have shown that an active enzyme is required to catalyze the exchange reaction. Boiled enzyme controls showed no exchange when compared to identical incubations containing active FBPase.

Fitting to the data for the Mn^{2+}-FBPase exchange reaction to Scheme II requires a $k_x/k_{off} = 2$ and a value for $k'_{on} = 3 \times 10^{-3}$ s^{-1}, where $k'_{on}/E = k_{on}$ is the second-order rate constant for formation of the exchange-competent E·fru-6-P·P_i complex.

Exchange in the Absence of Fru-6-P. A similar experiment was performed as above, with fru-6-P omitted from the reaction mixture. A very slight increase in P_0, with a corresponding decrease in P_3 and P_4, was observed over an 8-h time period. A $t_{1/2}$ for total oxygen exchange of 38 h was estimated, in comparison to a $t_{1/2}$ of 14 min for an identical reaction in which 200 mM fru-6-P was present—a 160-fold stimulation of the rate of overall ^{18}O exchange by this concentration of fru-6-P.

Dependence upon Fru-6-P Concentration. The dependence of the ^{18}O exchange reaction upon fru-6-P concentration was examined by determining the extent of ^{18}O loss at two time points for a series of reaction mixtures in which the fru-6-P concentration was varied from 0 to 500 μM. The exchange reaction shows saturation at approximately 20 μM fru-6-P. The fru-6-P concentration required for half-maximal exchange velocity is estimated at 5–10 μM, a range that compares favorably with the dissociation constants for the first two fru-6-P molecules bound per tetramer of 0.24 and 5.0 μM (Benkovic et al., 1978), and K_i = 0.8 μM for competitive product inhibition from steady-state kinetic studies (Dudman et al., 1978).

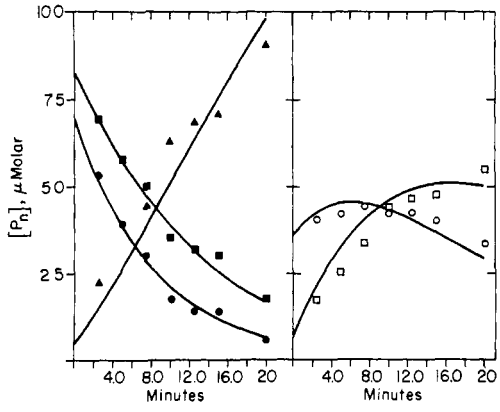


FIGURE 2: Data and the theoretical fit for Mn^{2+}-fru-6-P exchange. Simulation is that of an abbreviated Scheme II, with $k_{on} = 3 \times 10^{-3}$ s^{-1}, $k_{off} = 2.5$ s^{-1}, and $k_x = 5$ s^{-1}. The following initial concentrations observed from experimentation were used for the simulation: P_4 (●) = 70 μM; P_3 (■) = 83 μM; P_2 (○) = 36 μM; P_1 (□) = 7 μM; P_0 (▲) = 5 μM.

Dependence on the Nature of the Divalent Metal Ion. The exchange reaction was examined with 5 mM Mg^{2+} and 0.1 mM EDTA [the same conditions as the Mg^{2+} assay of Frey et al. (1977)]. Mg^{2+}–FBPase was also found to catalyze ^{18}O exchange out of [^{18}O]P_i, with a similar distribution pattern as that found for Mn^{2+}–FBPase. Fitting of the data to Scheme II has given a $k_x/k_{off} = 1.4$ and a value of $k'_{on} = 1.8 \times 10^{-4}$ s^{-1}. The enzyme concentration was 0.1 μM in active site.

Substitution of Methyl Fructoside 6-Phosphate for Fru-6-P. Data obtained from a reaction in which 0.2 mM (α + β)-methyl D-fructofuranoside 6-phosphate (Fishbein et al., 1974) was substituted for fru-6-P indicate that no exchange has occurred under these conditions.

Incorporation of ^{18}O into P_i during Hydrolysis of Fru-1,6-P_2. In order to compare the observed equilibrium exchange with the steady-state forward reaction, we performed experiments in which fru-1,6-P_2 was hydrolyzed by FBPase in [^{18}O]H_2O (initially 27–34 atom % ^{18}O). Introduction of ^{18}O from the solvent into P_i owing to the fru-6-P stimulated $H_2O \rightleftarrows P_i$ exchange was eliminated by trapping the fru-6-P produced with the glucose-6-phosphate isomerase–glucose-6-phosphate dehydrogenase coupling system.

The reaction investigated is described by Scheme III. The amount of ^{18}O incorporated into P_i from the solvent will be determined as before by the k_x/k_{off} ratio, limiting at $n = 1$ when $k_x/k_{off} << 1$ and $n = 4$ when $k_x/k_{off} >> 1$. Results of these experiments at low and high conversion to the product, as well as values predicted via computer simulation, are listed in Table II. For both Mg^{2+} and Mn^{2+}, the values of k_x/k_{off}

Scheme III

$$\text{fru-1,6-P}_2 + \text{E} \rightleftharpoons \text{E·fru-1,6-P}_2 \underset{k_x}{\overset{H_2{}^{18}O}{\rightleftharpoons}} \text{E·fru-6-P·P}_n \xrightarrow{k_{off}} \text{E} + \text{fru-6-P} + \text{P}_n$$

Table II: Incorporation of ^{18}O into P_i Produced during Hydrolysis of Fru-1,6-P_2

metal	atom % ^{18}O content: water	P_i produced	% of total	k_x/k_{off}[a]	% hydrolysis
Mg^{2+}	34.4	11.0	31.8	obsd	100
		11.0	31.8	0.44	
		10.4	30.2	0.33	
Mg^{2+}	27.4	6.7	24.5	obsd	12.5
		6.9	25.0	0.01	
Mn^{2+}	29.2	8.0	27.4	obsd	100
		8.3	28.4	0.20	
		7.8	26.8	0.10	

[a] "Obsd" refers to data obtained from experiments. Lines where a numerical value appears refer to predictions made by simulation.

are less than unity, in marked contrast to the fru-6-P stimulated $[^{18}O]P_i$ exchange. The higher values observed for k_x/k_{off} in the 100% hydrolysis experiments have been attributed to the fru-6-P dependent equilibrium exchange.

Discussion

The participation of a phosphoryl enzyme intermediate in the catalytic mechanism of FBPase has not been implicated to date. Physiological phosphorylation at other than the active site has been demonstrated (Riou et al., 1977) and attempts to implicate the regulatory nature of this phosphorylation were made (Riou et al., 1977; Mendicino et al., 1978). A catalytically significant phosphoryl enzyme intermediate has been demonstrated for a number of phosphotransferases—e.g., the alkaline phosphatases (Schwartz, 1963; Fernley, 1971), phosphoglucomutase (Anderson & Jolles, 1957), and glucose-1,6-diphosphate synthetase (Wong & Rose, 1976).

In optimal cases the existence of a catalytically significant phosphoryl enzyme can be demonstrated by an apparent association of ^{32}P label with a purifiable protein and the demonstration of the catalytic competence of such a species, although an apparent phosphoryl enzyme could be simply a tightly bound ligand (Walsh & Spector, 1971; Johnson et al., 1976). The demonstration of ping-pong kinetics (for two-substrate reactions) or of an exchange half-reaction becomes the only feasible probe with increasing instability of the phosphoryl enzyme. The $[^{18}O]P_i$ exchange reaction is an example of such an exchange process which possesses the additional advantage that the observation of exchange, in this case, depends only on the association and dissociation of a single ligand. In a two-substrate system, exchange may go undetected if dissociation of one reactant is slow relative to the catalytic steps involved in the exchange.

Patterns of $[^{18}O]P_i \rightleftarrows H_2O$ Exchange. Various values of k_x/k_{off} for ^{18}O exchange between P_i and H_2O have been observed. At pH 7.0, Eargle et al. (1977) have found for alkaline phosphatase that $k_x/k_{off} << 1.0$, indicating that dissociation is not rate limiting. Recently Bock & Cohn (1978) have shown that, as high as pH 10, the pattern of ^{18}O exchange for Zn^{2+}–alkaline phosphatase is also governed by $k_x/k_{off} << 1.0$. Their findings indicate that the dissociation of P_i from the enzyme is never slow with respect to the phosphorylation–dephosphorylation cycle of Zn^{2+}–alkaline phosphatase. However, with Co^{2+}–alkaline phosphatase at pH 6.8, their data show a $k_x/k_{off} = 3$, thus suggesting that either E·P_i dissociation has slowed or phosphorylation–dephosphorylation has increased to give this ratio.[6] The $[^{18}O]P_i$ exchange data alone cannot differentiate between these two alternatives.

An extreme case where k_{off} is very much slower than k_x has recently been reported by Webb et al. (1978) for ^{18}O exchange catalyzed by myosin subfragment 1. Although the overall ^{18}O exchange process is slow (requiring 15 h to achieve conversion to approximately 40% P_0), ^{18}O distribution analysis shows a constant ratio of P_2, P_3, and P_4 and no appearance of P_1 at all during the reaction. They estimate a k_x/k_{off} ratio of greater than 50 to explain these observtions. Simulations of Scheme II using k_x/k_{off} values of 50 and 100 (see Figure 1F) are entirely consistent with their description of the data. The pattern of ^{18}O exchange between P_i and H_2O catalyzed by FBPase is fit with intermediate values of $k_x/k_{off} \simeq 1.4$–2.0.

Fru-6-P Dependence of FBPase Catalyzed Exchange. Our data have been fitted to an abbreviation of Scheme II, in which k'_{on} represents the product of the association rate constant (k_{on}) and the concentration of that enzyme species involved in binding P_i. As noted above, the ^{18}O exchange rate is half-maximal at approximately 7 μM fru-6-P. By employment of an average binding constant of 1.4×10^5 M^{-1} for fru-6-P and P_i binding to two of the four active sites, the concentration of E·fru-6-P, at 1.1 μM total active sites of Mn^{2+}–FBPase and 200 μM each in fru-6-P and P_i, would be 5×10^{-8} M.[7] Since the binding of P_i to E·fru-6-P generates the species involved in ^{18}O exchange, the value of k_{on} is 6×10^4 M^{-1} s^{-1}. A similar analysis gives, for the Mg^{2+}–enzyme, a value for k_{on} of 2×10^3 M^{-1} s^{-1}. If association were to be diffusion-limited, a value of approximately 10^8 M^{-1} s^{-1} would be expected for interaction of a protein with a small molecule.

This slow association rate suggests that P_i does not bind to directly produce the exchange-competent complex in either the Mn^{2+} or the Mg^{2+} systems but instead binds to form a complex which must in turn undergo a conformational change before exchange can occur. Since the concentration of P_i employed likewise should be effectively saturating for exchange, the first-order rate constant for this process may be estimated as ca. 10^{-3} s^{-1}. Similarly, the rate coefficient for formation of the catalytically competent enzyme·fru-1,6-P_2 complex has been estimated to be approximately 10^7 M^{-1} s^{-1} (Caperelli et al., 1978), suggesting that "binding" of fru-1,6-P_2 prior to hydrolysis is also a multistep process involving binding and conformational change(s).

An analogous situation has been proposed in the myosin subfragment 1 system. The rate-determining step in ATP hydrolysis by this protein is proposed to be a slow isomerization of two protein·ADP·P_i complexes. Data from rapid fluorescence kinetics measurements (Bagshaw & Trentham, 1974; Bagshaw et al., 1974) have implicated this event. An estimate of the rate constants for this interconversion has been extracted from the $[^{18}O]P_i$ exchange data for this system (Webb et al., 1978) and is consistent with the proposed slow isomerization of protein–product complexes.

Equilibrium vs. Steady-State $[^{18}O]P_i \rightleftarrows H_2O$ Exchange. The marked stimulation of the $[^{18}O]P_i \rightleftarrows H_2O$ exchange by fru-6-P but not the inhibitor methyl D-fructofuranoside 6-phosphate suggests that the enzyme intermediate(s) responsible

[6] Bock and Cohn used the treatment of Boyer et al. (1977) to determine an R value of 3 for the Co^{2+}–alkaline phosphatase exchange data. We have simulated that same data with our Scheme II and obtain $k_x/k_{off} = 3$.

[7] Equilibrium binding experiments with P_i and fru-6-P employing Mn^{2+}– and Mg^{2+}–FBPase revealed that the interaction between binding sites is negatively cooperative for both ligands with the two initial binding sites characterized by constants that are 1–2 orders of magnitude larger than those for the third and fourth sites (Benkovic et al., 1978).

for exchange lies on the catalytic pathway but does not reveal their order, if any, of formation. It is also probable that the very slow exchange observed in the absence of added fru-6-P is caused by trace amounts of tightly bound fru-6-P. As noted above, the pattern of the [^{18}O]P_i exchange in the presence of fru-6-P can be described by a partition ratio of $k_x/k_{off} \simeq$ 1.4–2.0. However, this ratio decreases to 0.01 for the hydrolysis of fru-1,6-P_2 by FBPase in the presence of [^{18}O]H_2O, in accord with the single ^{18}O atom substitution required by P–O cleavage. It is important to realize that the k_x/k_{off} ratio reflects the partitioning minimally of an intermediate enzyme species represented above as E·fru-6-P·P_i. Thus, despite differences in fru-6-P levels between the steady-state hydrolysis experiments and those of equilibrium exchange, the measurement of k_x/k_{off} only depends on the presence of E·fru-6-P·P_i, and its value is not dependent on fru-6-P levels. If the intermediate(s) responsible for exchange and hydrolysis was identical, then the measures of k_x/k_{off} should be equal for both experiments.

Two explanations may be offered for the observed inequivalence: first, that this exchange process, despite its dependence on fru-6-P, is simply not related to the hydrolysis and second, that the enzyme intermediates in the two sets of experiments are related but not identical. Given the fact that FBPase is tetrameric, a key factor may be the state and nature of occupancy of the four active sites. In the case of the hydrolysis reaction, both rapid quench (Caperelli et al., 1978) and pre-steady-state experiments (Benkovic et al., 1979) have shown that all four subunits are occupied and turn over at fru-1,6-P_2 concentrations in accord with an average binding constant of less than 1 μM. In the case of the exchange reaction, binding studies of fru-6-P or P_i in the presence of Mn^{2+} and Mg^{2+} have shown pronounced negative cooperativity, so that only two of the four sites would be occupied under exchange conditions. Since P_i and fru-6-P binding is not exclusive, two sites would remain vacant. Thus, in a catalytic cycle commencing with fru-1,6-P_2 (>1 μM), species $\sum$E·(fru-1,6-P_2)$_{4-n}$(fru-6-P·P_i)$_n$ would be present but the species (E·fru-6-P·P_i)$_2$ would not accumulate until the fru-1,6-P_2 was exhausted. In terms of $n = 4$, hydrolysis proceeds via

$$\text{E·(fru-1,6-P}_2)_4 \underset{k'_x}{\overset{^{18}O}{\rightleftharpoons}} \text{E·(fru-6-P·P}_i)_4 \xrightarrow{k'_{off}}$$

but exchange proceeds via

$$\text{E·(fru-6-P·P}_i)_2 \underset{^{18}O}{\overset{(k_x}{\rightleftharpoons}} \text{E·(fru-6-P·P}_i^{n-1})_2 \xrightarrow{k_{off}}$$

If the nature and degree of occupancy affect k_{off} (k'_{off}) relative to k_x (k'_x), then it is possible to alter affect extent of ^{18}O exchange observed in the two sets of experiments yet have the same intermediates involved. Alternatively, k_{off} and k_x may not be affected by the presence of fru-1,6-P_2, but the rotation of the P_i produced upon hydrolysis might be restricted during steady-state FBPase catalyzed processes. In essence, the appearance of E·(fru-6-P·P_i)$_2$ in the steady-state experiments would not occur until fru-1,6-P_2 is substantially depleted so that a k_x/k_{off} ratio characteristic of this species alone would not be observed in steady-state experiments. The differing magnitudes of the rate parameters, e.g., k_{off} (k'_{off}), according to this rationale suggest that cooperative effects between sites may influence the rate processes associated with the exchange phenomenon.

Chemical Nature of the [^{18}O]$P_i \rightleftarrows H_2O$ Exchange. The nature of the ^{18}O exchange reaction likewise has two rationales. Mixtures of FBPase, P_i, and fru-6-P, under these conditions, could synthesize fru-1,6-P_2 by a net reversal of the hydrolytic reaction. Subsequent hydrolysis of the fru-1,6-P_2 formed, without its escape from the active site, would supply the chemical events required to explain the ^{18}O exchange and still be consistent with the observed "irreversible" nature of the FBPase reaction. Alternatively, binding of the second ligand, fru-6-P, at the active site of FBPase could induce a conformational change required to assemble the active-site residues in the proper conformation necessary for phosphorylation of the enzyme by P_i. Given the validity of this hypothesis, one might predict that analogues of fru-6-P should substitute as the necessary second ligand, thus promoting the ^{18}O exchange reaction without requiring the formation of fru-1,6-P_2 in the active site. Although tests are still in progress to conclusively rule out this hypothesis, the absence of ^{18}O exchange upon substitution of ($\alpha + \beta$)-methyl D-fructofuranoside 6-phosphate for fru-6-P tends to discredit it, even though ($\alpha + \beta$)-methyl fructofuranoside 6-phosphate has been shown to be a competitive inhibitor of fru-1,6-P_2 hydrolysis. These results therefore lead us to tentatively conclude that the chemical event which must be postulated to explain the ^{18}O exchange reaction is reversibility, leading to cyclic formation and hydrolysis of fru-1,6-P_2 within the enzyme active site.

Comparison of This Theoretical Treatment with Others. The most recent other theoretical treatment of ^{18}O exchange which deals with the possible multiplicity of labeling of P_i is that of Boyer et al. (1977) [see also Hackney & Boyer (1978)]. Their expressions are derived based upon probability theory. The R factor used by these workers, and also utilized by Bock & Cohn (1978), is analogous to our k_x/k_{off} partition coefficient.[6] Our approach permits an easier intuitive assessment of our partition coefficient by visual examination of experimental data. However, both treatments are equally accurate, are complementary, and arrive at similar conclusions along independent lines of thought.

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