Koppel, D. (1972) *J. Chem. Phys. 57*, 4814-4820.
Kratky, O., Leopold, H., & Staubinger, H. (1973) *Methods Enzymol. 27D*, 98.
Kuo, T.-T., Huang, T.-C., & Chow, T.-Y. (1969) *Virology 39*, 548-560.
Makowski, L., & Caspar, D. L. D. (1978) in *The Single-Stranded DNA Phages* (Denhardt, D. T., Dresser, D., & Ray, D. S., Eds.) pp 626-643, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Makowski, L., & Caspar, D. L. D. (1981) *J. Mol. Biol. 45*, 611-617.
Marzec, C. J., & Day, L. A. (1980) *Biophys. J. 32*, 240-242.
Nave, C., Brown, R. S., Fowler, A. G., Ladner, J. E., Marvin, D. A., Provencher, S. W., Tsugita, A., Armstrong, J., & Perham, R. N. (1981) *J. Mol. Biol. 149*, 675-707.
Newman, J., Swinney, H. L., Berkowitz, S. A., & Day, L. A. (1974) *Biochemistry 13*, 4832-4838.
Newman, J., Swinney, H. L., & Day, L. A. (1977) *J. Mol. Biol. 116*, 593-606.
Pecora, R. (1964) *J. Chem. Phys. 40*, 1604-1614.
Stanisich, V. A. (1974) *J. Gen. Microbiol. 84*, 332-342.
Timasheff, S. N. (1970) in *Handbook of Biochemistry* (Sober, H. A., Ed.) 2nd ed., p C67, Chemical Rubber Co., Cleveland, OH.
Tirado, M. M., & Garcia de la Torre, J. (1979) *J. Chem. Phys. 71*, 2581-2587.
Tirado, M. M., & Garcia de la Torre, J. (1980) *J. Chem. Phys. 73*, 1986-1993.
Wiseman, R. L., & Day, L. A. (1977) *J. Mol. Biol. 116*, 607-611.

Rapid-Quench and Isotope-Trapping Studies on Fructose-1,6-bisphosphatase†

Jubrail F. Rahil, Margaret M. de Maine,‡ and Stephen J. Benkovic*

ABSTRACT: Rapid-quench kinetic measurements yielded pre-steady-state rate data for rabbit liver fructose-1,6-bisphosphatase (FBPase) (a tetramer of four identical subunits) that are triphasic: the rapid release of P_i (complete within 5 ms), followed by a second reaction phase liberating additional P_i that completes the initial turnover of two or four subunits of the enzyme (requiring 100-150 ms), and a steady-state rate whose magnitude depends on the [α-Fru-1,6-P_2]/[FBPase] ratio. With Mg^{2+} in the presence of excess α-fructose 1,6-bisphosphate (α-Fru-1,6-P_2) all four subunits turn over in the pre steady state; with Mn^{2+} only two of the four are active. Thus the expression of half-site reactivity is a consequence of the nature of the metal ion and not a subunit asymmetry. In the presence of limiting α-anomer concentrations only two of the four subunits now remain active with Mg^{2+} as well as with Mn^{2+} in the pre steady state. However, so that the amount of P_i released can be accounted for, a $\beta \rightarrow \alpha$ anomerization or direct β utilization is required at the active site of one subunit. Such behavior is consistent with the two-state conformational hysteresis displayed by the enzyme and altered affinities manifested within these states for α and β substrate analogues. Under these limiting conditions the subsequent steady-state rate is limited by the $\beta \rightarrow \alpha$ solution anomerization. These data in combination with pulse-chase experiments permit evaluation of the internal equilibrium, which in the case of Mg^{2+} is unequivocally higher in favor of product complexes and represents a departure from balanced internal substrate-product complexes.

Fructose-1,6-bisphosphatase (EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase, FBPase[1]) (Benkovic & de Maine, 1982) catalyzes the hydrolysis of D-fructose 1,6-bisphosphate (Fru-1,6-P_2) to D-fructose 6-phosphate (Fru-6-P) and inorganic phosphate (P_i). It is a tetrameric protein with four identical subunits (Traniello et al., 1971) and requires Mg^{2+}, Mn^{2+}, Zn^{2+}, or Co^{2+} for its catalytic activity. Kinetic studies with Mn^{2+} on the rabbit liver enzyme demonstrated its high stereospecificity for the α anomer of Fru-1,6-P_2 (Frey et al., 1977). However, the stereospecificity appears anomalous in that the enzyme binds both the α and β anomers of methyl D-fructofuranoside 1,6-bisphosphate (K_i = 7.2 μM and 1.7 μM, respectively) (de Maine & Benkovic, 1972). An explanation is provided by the existence of hysteretic active and resting forms of FBPase (de Maine & Benkovic, 1979). Results from pre-steady-state kinetic investigations employing a phenol red indicator method on FBPase with Mg^{2+} fitted the proposed hysteresis. They suggested a reaction sequence in which binding of the substrate is followed by a first-order conformational change prior to the establishment of the steady state. The conformational change (manifest by proton release) is followed by a rapid proton uptake of a magnitude corresponding to the release of P_i from a molecule of Fru-1,6-P_2 by each subunit of the FBPase. After breakdown of the remaining substrate at the steady-state rate the catalytic cycle ends with the decay of the enzyme to its initial unreactive state (Benkovic et al., 1979).

The present study concerns itself with molecular events that occur in the transient phase, namely, (1) verification that P_i is indeed released in this phase, (2) the stoichiometry of P_i release relative to conditions where Fru-1,6-P_2 as the α anomer is less than FBPase subunit concentration, (3) the influence

† From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received December 1, 1981.* This investigation was supported by a grant from the National Institutes of Health, U.S. Public Health Service (GM 13306).
‡ Awardee of an Eloise Gerry fellowship from Sigma Delta Epsilon.

[1] Abbreviations: FBPase, fructose-1,6-bisphosphatase; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

0006-2960/82/0421-3358$01.25/0

of various divalent metal ions on the events in the transient phase, and (4) the internal equilibrium between Fru-1,6-P_2 and Fru-6-P plus P_i at the FBPase active site.

Experimental Procedures

Materials

Fru-1,6-P_2, Fru-6-P, $NADP^+$, EDTA, phosphohexose isomerase, glucose-6-phosphate dehydrogenase, and phosphofructokinase were purchased from Sigma Chemical Co. Inorganic salts were reagent grade. Tris buffers were prepared from Trizma base (Sigma, reagent grade). Liquiscint scintillation cocktail was obtained from National Diagnostics. Young fasted rabbit livers were purchased from Pel-Freez Biologicals. Carrier-free [γ-^{32}P]ATP (500 μCi), obtained from New England Nuclear, was reacted with Fru-6-P and phosphofructokinase to make [1-^{32}P]Fru-1,6-P_2, which was purified by chromatography on a Dowex 1-Cl column (Bartlett, 1959).

Methods

FBPase. Neutral rabbit liver FBPase was purified from the frozen livers of young, 24 h fasted rabbits by the procedure of Ulm et al. (1975) as modified by S. J. Benkovic et al. (1974). One sharp protein band was observed for the purified enzyme in both disc and sodium dodecyl sulfate gel electrophoresis as described previously (Benkovic, P. A., et al., 1974). Protein concentrations were determined on the basis of the standard absorbance of 0.71 for a 1.0 mg/mL solution.

The enzyme was assayed spectrophotometrically by following the rate of NADPH production at 340 nm in the presence of excess phosphohexose isomerase and glucose-6-phosphate dehydrogenase. The routine assay solution for the activity at 25 °C contained 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM $NADP^+$, 7.6 units of phosphohexose isomerase, 1.7 units of glucose-6-phosphate dehydrogenase, and 0.1 mM Fru-1,6-P_2 in a total volume of 1 mL. When Mn^{2+} was utilized as the cofactor, 0.5 mM $MnCl_2$ was used with no EDTA.[2] This assay provided a rate that yielded the steady-state rate when adjusted for the higher enzyme concentration in the rapid-quench systems.

Extraction Analysis of [^{32}P]P_i. Inorganic phosphate was separated from the sugar phosphates by adapting the Jencks & Gilchrist (1964) modification of the Martin & Doty (1949) procedure. A known volume of the quenched reaction solution (0.2 mL) was added to 0.3 mL of water-saturated isobutyl alcohol. The mixture was vortexed for 30 s. To this mixture was added 0.3 mL of a solution made from 2 g of ammonium molybdate, 5.55 mL of concentrated H_2SO_4, and water to make 100 mL. After a vigorous vortexing for 60 s the two layers were separated by low-speed centrifugation. Equal volumes (150 μL) of each layer were added to 3 mL of Liquiscint scintillation cocktail. ^{32}P radioactivity was measured with a Beckman LS 8100 liquid scintillation spectrometer.

Comparison of the radioactivity in the organic phase to that of the aqueous gave the relative distribution of ^{32}P as P_i and as Fru-1,6-P_2. Measured values of radioactivity were adjusted for the actual volumes of the two layers. The counts of the organic layer (containing the [^{32}P]P_i) were further corrected for trace P_i in the Fru-1,6-P_2 and for extraction efficiency. The >90% recovery of radioactivity permitted the calculation of the P_i molar concentrations from the number of counts in the P_i phase and the specific activity of the original sugar solution.

Rapid-Quench Experiments. The rapid-quench kinetic experiments were peformed with a Durrum multimixer apparatus as modified in this laboratory (Benkovic, P. A., et al., 1974). Runs were performed at ambient temperature by mixing equal volumes of enzyme–metal and Fur-1,6-P_2–metal solutions, followed by rapid quenching with twice the reaction volume of 5% $HClO_4$. Approximately 0.25–0.35 mL of quenched solution was collected. As a precaution against systematic unrecognized error, such as enzyme denaturation, the reaction times were randomly varied during the course of the experiment. The stock enzyme solutions contained 2–16 μM FBPase, 5 mM $MgCl_2$, and 0.1 mM EDTA (or 0.5 mM $MnCl_2$ and no EDTA) in 50 mM Tris-HCl at pH 7.6. In the substrate stock solutions the FBPase was replaced by 40–400 μM [1-^{32}P]Fru-1,6-P_2. The quenched reaction mixtures were stored at 0 °C until assayed as described above. The extent of reaction for each time point was determined by calculating the P_i concentration from the radioactivity of the assay layers.

Pulse–Chase Experiments. These experiments were performed by mixing 0.1 mL of enzyme–metal and 0.1 mL of [1-^{32}P]Fru-1,6-P_2–metal solutions as described above for the rapid-quench experiments. After a preselected reaction time (typically 25 or 250 ms) there was added 0.2 mL of unlabeled Fru-1,6-P_2–metal cofactor solution (chase stop). After times of 3–10 s the solutions were quenched manually by adding them to 0.2 mL of 10% $HClO_4$, followed immediately by vigorous vortexing. So that correction for [^{32}P]P_i released after the dilution step (chase stop) could be made, the product concentration obtained at different quench times was extrapolated to zero time.[3] The [^{32}P]P_i cpm were measured and corrected as described above.

Results

Control Experiments. Proper execution of rapid-quench and pulse–chase experiments demands efficient mixing and quenching of active enzyme solutions to prevent artifacts (Rose, 1980). Control experiments were conducted by using different concentrations of the quenching acid (5%, 10%, and 20% $HClO_4$) to determine whether the amount of product (P_i) trapped was determined by the acid level. No dependency was noted, thus indicating efficient mixing and sufficient acid to stop instantaneously—on our time scale—FBPase turnover. Similarly, pulse–chase experiments were performed with the concentration of the chase solution being 1, 2, and 4 mM Fru-1,6-P_2. The amount of P_i was independent of the Fru-1,6-P_2 concentration in the chase. Moreover, in the converse experiment when labeled [1-^{32}P]Fru-1,6-P_2 was used as the chase and unlabeled substrate as the reactant, no [^{32}P]P_i was detected upon extrapolation to zero time. Further control experiments found insignificant (less than 3% in 4 h) spontaneous hydrolysis of the [1-^{32}P]Fru-1,6-P_2 at 0 °C in the quenching acid and under assay conditions. The [^{32}P]P_i assay method gave 90% or more recovery of the original radioactivity, with greater recovery for Mn^{2+} systems than for Mg^{2+}.

Acid-Quench and Pulse–Chase Protocols. The amount of accumulated product, $(P_A)_t$, upon acid quenching of the active enzyme at time t is presumed equivalent to the concentration

[2] The pronounced activation of the Mg^{2+} assay by EDTA has been attributed to the removal of inhibitory Zn^{2+} through chelation (Nimmo & Tipton, 1975). In contrast EDTA at 0.5 mM Mn^{2+} has a negligible effect (<10%) on activity (Dudman et al., 1978) in accord with the apparent higher affinity of Mn^{2+} vs. Mg^{2+} for FBPase (Benkovic et al., 1978b) and the former's ability to displace Zn^{2+} from its structural sites. Consequently EDTA is omitted from the Mn^{2+} assays.

[3] In practice infinite dilution of the labeled substrate is not feasible. So that correction for the P_i formed during the chase interval could be made, the time for addition of acid was varied (typically 3, 5, 7, and 10 s). Extrapolation of [^{32}P]P_i (as cpm or percent) to zero time gives the [^{32}P]P_i present at the chase time; i.e., the intercept is $(P_C)_t$.

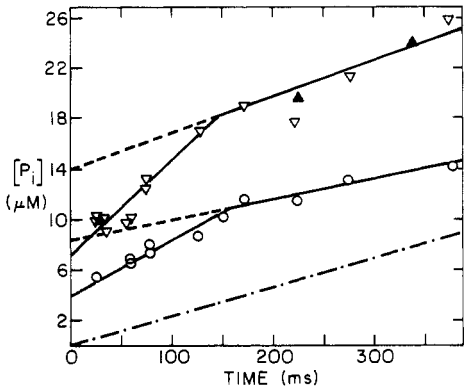

FIGURE 1: Plots of $[P_i]$ formed vs. time for FBPase·Mg^{2+} rapid quench at (▽) 4 μM FBPase and 200 μM [1-^{32}P]Fru-1,6-P_2 and at (○) 2 μM FBPase and 200 μM [1-^{32}P]Fru-1,6-P_2. The reaction solution contained 5 mM $MgCl_2$, 0.1 mM EDTA, and 50 mM Tris-HCl, pH 7.6. (▲) denotes pulse–chase experiments for the system denoted by (▽). The chase solution contains 2 mM Fru-1,6-P_2. The broken line (-·-) corresponds to the steady-state rate measured at low enzyme concentration via the coupled spectrophotometric assay.

of free [^{32}P]P_i in solution (resulting from net enzyme turnover) plus the amount that is bound to enzyme (eq 1). (The metal ion is omitted for ease of expression.)

$$E + [1\text{-}^{32}P]\text{Fru-1,6-}P_2 \rightleftharpoons E\cdot[1\text{-}^{32}P]\text{Fru-1,6-}P_2 \xrightleftharpoons{K_{int}} E\cdot\text{Fru-6-P}\cdot[^{32}P]P_i \rightleftharpoons E + \text{Fru-6-P} + [^{32}P]P_i$$

$$(P_A)_t = [E\cdot\text{Fru-6-P}\cdot[^{32}P]P_i]_{ss} + [^{32}P]P_i \quad (1)$$

The bound P_i is found primarily in the steady-state ternary E·Fru-6-P·P_i complex since the K_i for P_i in the presence of Mn^{2+} is 2.7 mM (Dudman et al., 1978). With Mg^{2+} the binding of P_i to FBPase is at least 100-fold less (Benkovic et al., 1978a).

The typical kinetic behavior of FBPase turnover under conditions of excess α-Fru-1,6-P_2 in the presence of Mg^{2+} is shown in Figure 1. Extrapolation of the steady-state part of the curve to zero time corrects for [^{32}P]P_i released in that phase and gives an intercept equivalent to the steady-state concentration of the ternary complex E·Fru-6-P·[^{32}P]P_i, i.e.

$$(P_A)_{t=0} = [E\cdot\text{Fru-6-P}\cdot[^{32}P]P_i]_{ss} \quad (2)$$

Alternatively, the enzymatic reaction with labeled substrate can be "effectively" stopped by diluting the labeled substrate with a large concentration of unlabeled Fru-1,6-P_2, followed by acid quenching (chase stop). Under these conditions the enzyme will hydrolyze a fraction of the unreacted bound [1-^{32}P]Fru-1,6-P_2 (dependent on the partitioning of E·Fru-1,6-P_2 between free substrate and product), thus adding [^{32}P]P_i to the product pool. Sufficient dilution by nonradioactive chase prevents turnover of unbound [1-^{32}P]Fru-1,6-P_2.[3] When dissociation of Fru-1,6-P_2 from the enzyme is assumed negligible (Caperelli et al., 1978) so that E·Fru-1,6-P_2 is converted totally to products, the amount of P_i product trapped by chase stop, $(P_C)_t$, equals $(P_A)_t$ plus the steady-state concentration of the E·Fru-1,6-P_2 complex. A linear extrapolation of $(P_C)_t$

$$(P_C)_t = [E\cdot[1\text{-}^{32}P]\text{Fru-1,6-}P_2]_{ss} + [E\cdot\text{Fru-6-P}\cdot[^{32}P]P_i]_{ss} + [^{32}P]P_i \quad (3)$$

vs. time to zero time corrects for the released [^{32}P]P_i so that $(P_C)_{t=0}$ is a measure of the substrate and product enzyme complexes (eq 4).

$$(P_C)_{t=0} = [E\cdot[1\text{-}^{32}P]\text{Fru-1,6-}P_2]_{ss} + [E\cdot\text{Fru-6-P}\cdot[^{32}P]P_i]_{ss} \quad (4)$$

Table I: FBPase·Mg^{2+} Rapid-Quench Results[a]

[FBPase][b] (μM)	[[1-^{32}P]Fru-1,6-P_2][c] (μM)	intercept[d] at $t = 0$ (μM)	rate[d] (μM/s)
2	200	4.0	43
		8.4	19
4	200	7.2	74
		14.0	33
4	40	4.6	80
		9.4	43
4	20	4.0	65
		7.8	30

[a] The upper line of each entry belongs to the second phase of the rate plot while the lower line belongs to the steady-state part (or the third phase). [b] Determined spectrophotometrically at 280 nm on the basis of the standard absorbance of 0.71 for 1.0 mg/mL. [c] Determined by the coupled spectrophotometric assay for FBPase described in the text. [d] Determined by using linear regression least-squares analysis.

For evaluation of the internal equilibrium constant (K_{int}) the concentration of the E·[1-^{32}P]Fru-1,6-P_2 complex is obtained from the difference (Δ) between $(P_C)_{t=0}$ and $(P_A)_{t=0}$ (Wilkinson & Rose, 1979). Since

$$K_{int} = [E\cdot\text{Fru-6-P}\cdot[^{32}P]P_i]/[E\cdot[1\text{-}^{32}P]\text{Fru-1,6-}P_2]$$

and

$$\Delta = (P_C)_{t=0} - (P_A)_{t=0} = [E\cdot[1\text{-}^{32}P]\text{Fru-1,6-}P_2]_{ss}$$

then

$$K_{int} = (P_A)_{t=0}/\Delta \quad (5)$$

The kinetic behavior of FBPase was studied by employing the experimental strategies described above. Although these experiments require the use of relatively large enzyme concentrations (2–8 μM), the enzyme should be saturated at all of the substrate concentrations studied (20–200 μM). Computer calculations using published values of the binding constants for the stepwise addition of substrate to the four subunits of the enzyme [K_1, K_2, K_3, and K_4 being 2.9×10^7 M^{-1}, 5.8×10^5 M^{-1}, 7.8×10^4 M^{-1}, and 4.8×10^4 M^{-1}, respectively (Libby et al., 1975)] support the above assumption that "all enzyme" was bound to substrate at zero time. We presume that this calculation applies to both the α- and β-anomeric forms since the K_i values are similar for α and β analogues that act as competitive inhibitors (de Maine & Benkovic, 1972). The experimental results will be presented separately for Mg^{2+} and Mn^{2+}.

FBPase·Mg^{2+} Enzyme. With substrate in excess relative to enzyme (200 μM Fru-1,6-P_2 vs. 4 and 2 μM FBPase) 4 equiv of P_i/equiv of enzyme is released rapidly (Figure 1). A total of 2 μM P_i/μM FBPase forms within the dead time of the instrument ($\lambda_1 > 70$ s^{-1}), followed by an additional 2 equiv of P_i with $\lambda_2 = 15.6$ s^{-1}. At greater than 150 ms the observed rate compares favorably to that obtained at assay enzyme concentrations (ca. 0.01 μM). Extrapolation of the steady-state segments to zero time yields intercepts of 14.0 and 8.4 μM for 4 and 2 μM FBPase (16 and 8 μM in sites) (Table I).

With the Fru-1,6-P_2 concentration at 40 μM the available α anomer (Benkovic et al., 1972) (8 μM) is less than the FBPase subunit concentration (16 μM). Under these conditions extrapolation of the steady-state rate to time zero yields an intercept on the $[P_i]$ axis of ca. 9.4 μM (Figure 2). This value is approximately half that observed at 200 μM Fru-1,6-P_2 (Table I) and is approximately the concentration of the α anomer. However, a release of 4 μM P_i/16 μM FBPase

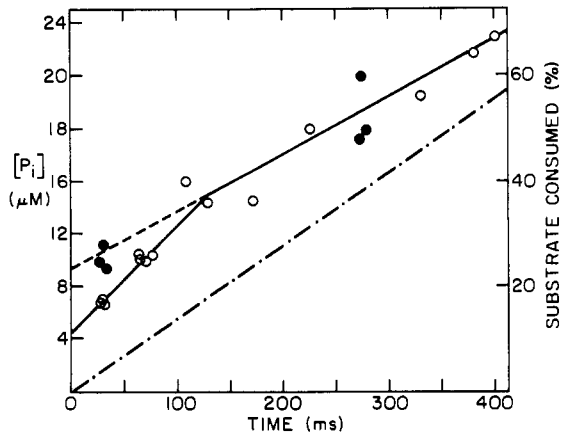

FIGURE 2: [P_i] formed at indicated times for rapid-quench (○) and pulse–chase (●) experiments performed at 4 μM FBPase, 40 μM [1-^{32}P]Fru-1,6-P_2, 5 mM $MgCl_2$, and 0.1 mM EDTA in 50 mM Tris-HCl at pH 7.6. The steady-state rate is denoted by (---).

Table II: Pulse–Chase and Rapid-Quench Parameters[a]

metal cofactor	[Fru-1,6-P_2] (μM)	FBPase (μM)	$(P_A)_{t=0}$[b] (μM)	$(P_C)_{t=0}$[c] (μM)	Δ (μM)
Mg^{2+}	200	4.0	14.0	14.0	0
	90	4.0	14.0	14.0	0
	40	4.0	9.4	9.4	0
Mn^{2+}	200	8.0	17.6	30.5	12.9
	82	8.0	17.6	30.5	12.9

[a] Parameters defined under Results (eq 2, 4, and 5). Includes only experiments carried out at the steady-state stage of the reaction. [b] Obtained by extrapolation to zero time of the steady-state portion of the rate plot of P_A vs. time. [c] Obtained by extrapolation to zero reaction time of the straight line that passes through the chase points and maintains the same steady-state rate as that obtained by rapid quench.

subunits still is observed within the dead time of the instrument.

Further decreasing the substrate concentration to 20 μM but maintaining the FBPase at 4 μM yields a biphasic rate plot (with a nonzero intercept) nearly identical with the one observed at 40 μM. Since the equilibrium α-anomer concentration is ca. 4 μM, the $(P_A)_{t=0}$ value of 7.8 μM (Table I) suggests that the enzyme is partially able to utilize β-Fru-1,6-P_2.

The values of $(P_C)_t$ at 40, 90, and 200 μM Fru-1,6-P_2 at a reaction time of 226 ms overlapped with the rapid-quench values (Figures 1 and 2). Extrapolation to $t = 0$ employing the steady-state rate achieved at times >100 ms yields the values for $(P_C)_{t=0}$ listed in Table II. The values of $(P_C)_t$ at 29 ms are coincident with $(P_A)_t$ for 200 μM Fru-1,6-P_2 but higher by ca. 4 μM at 40 μM (Figures 1 and 2).

FBPase-Mn^{2+} Enzyme. The triphasic kinetic behavior also occurs in the presence of Mn^{2+}, although the burst magnitudes are attenuated. At excess substrate concentration (200 μM Fru-1,6-P_2 vs. 8 μM FBPase), ca. 8 μM P_i is generated within the dead time of the instrument and an additional 8–9 μM P_i is formed before the steady-state rate is attained. An identical reaction course was observed with an 82 μM Fru-1,6-P_2 concentration (Figure 3, Table III). Thus 2 equiv of Fru-6-P/FBPase is formed prior to the steady-state stage, although only 16 μM α anomer is available at equilibrium for 82 μM total Fru-1,6-P_2. The rate attained at >100 ms is the expected steady-state turnover rate.

In the above two runs, the α-anomeric specificity of the enzyme was not expressed. So that the supply of the α anomer could be limited, the substrate concentration was decreased to 41 μM while maintaining FBPase at 8 μM. The steady-state rate observed under these conditions was 34 μM/s (Figure 4, Table III), which is comparable to the expected β

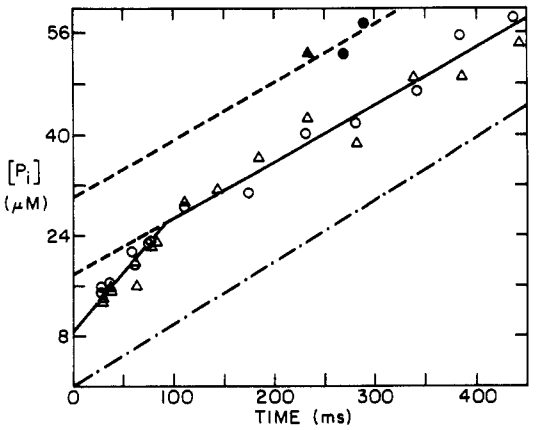

FIGURE 3: [P_i] formed at indicated times for FBPase-Mn^{2+} enzyme rapid-quench (open symbols) and pulse–chase (closed symbols) experiments at 8 μM FBPase and (○) 200 μM [1-^{32}P]Fru-1,6-P_2 or (△) 82 μM [1-^{32}P]Fru-1,6-P_2 in the presence of 0.5 mM $MnCl_2$ in 50 mM Tris-HCl at pH 7.6. The chase solutions contain (●) 2 and (▲) 1.4 mM Fru-1,6-P_2. The broken line (---) corresponds to the steady-state rate determined at low enzyme concentration via the coupled spectrophotometric assay.

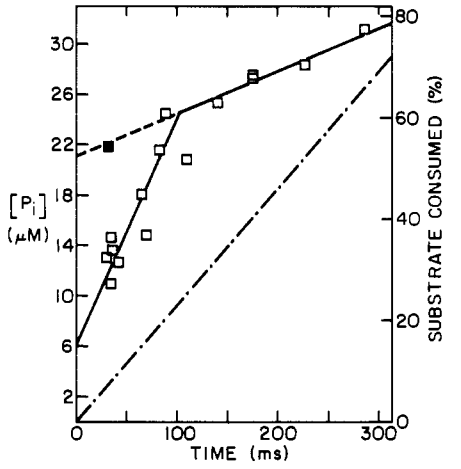

FIGURE 4: [P_i] formed at indicated times for pulse–chase (■) and rapid-quench (□) experiments at 8 μM FBPase, 41 μM [1-^{32}P]Fru-1,6-P_2, and 0.5 mM $MnCl_2$ in 50 mM Tris-HCl at pH 7.6. The concentration of Fru-1,6-P_2 in the chase solution contains 2 mM Fru-1,6-P_2. The broken line (---) corresponds to the steady-state rate determined at low enzyme concentration via the coupled spectrophotometric assay.

Table III: FBPase-Mn^{2+} Rapid-Quench Results[a]

[FBPase][b] (μM)	[[1-^{32}P]Fru-1,6-P_2][c] (μM)	intercept[d] at $t = 0$ (μM)	rate[d] (μM/s)
8	200	8.6	186
		17.6	93
8	82	8.6	186
		17.6	93
8	41	6.2	178
		21.0	34

[a] The upper line of each entry belongs to the second phase of the rate plot, while the lower line belongs to the steady-state part (or the third phase). [b] Determined spectrophotometrically at 280 nm on the basis of the standard absorbance of 0.71 for 1.0 mg/mL. [c] Determined by the coupled spectrophotometric assay for FBPase, described in the text. [d] Determined by using linear regression least-squares analysis.

→ α anomerization rate, 23 μM/s. In contrast the steady-state rate (measured via the coupled enzyme assay) is 96 μM/s. This expression of stereospecificity is in agreement with previous results (Frey et al., 1977). Despite the limiting α-anomer concentration, the enzyme again turns over in a biphasic manner approximately two molecules of Fru-1,6-P_2 per FBPase in its initial turnover since $(P_A)_{t=0}$ = 21 vs. 32 μM FBPase subunits.

The pulse–chase results at 288 ms are identical for the 200 and 82 μM substrate. The amount of P_i detected is increased by a factor of 1.6, which translates to a concentration of 30.5 μM P_i, signifying the total occupancy of the four FBPase sites at the steady state (Figure 3). Pulse–chase experiments performed at t = 30 ms before the steady state was achieved detected ca. 1.2 μM Fru-1,6-P_2/μM FBPase that is converted to P_i before dissociation. Due to experimental difficulties no chases were done during the steady-state stage of the reaction.

Discussion

Several distinct features characterize the kinetic behavior of FBPase under our various conditions. All product (P_i) vs. time plots (Figures 1–4) exhibit three regions: a very fast reaction segment ($\lambda > 70$ s^{-1}) that is complete within 5 ms, a second reaction phase liberating additional P_i that completes the initial turnover of two or four subunits of the enzyme and requires 100–150 ms, and a steady-state rate whose magnitude depends on the [α-Fru-1,6-P_2]/[FBPase] ratio.

In the presence of excess Fru-1,6-P_2 where the concentrations of the α and β anomers are greater than the FBPase subunit levels, the timing and amount of P_i liberated in the pre-steady-state phases depend on the nature of the metal ion. With Mg^{2+} there is an initial rapid turnover of ca. two of the FBPase subunits, liberating P_i within the dead time of the apparatus, followed by the release of P_i from the remaining two subunits within 150 ms (Figure 1 and Table I). Thus all four subunits are active during the initial turnover, consistent with their saturation by Fru-1,6-P_2. This behavior is in accord with pre-steady-state measurements made in the presence of Mg^{2+} by an indicator method that found the release of ca. 4 hydroxide equiv/mol of FBPase over the same time period (Benkovic et al., 1979). With Mn^{2+} the amounts of P_i liberated are halved: only one P_i is formed per FBPase at $t <$ 5 ms, and only one additional P_i is liberated per FBPase during the remainder (up to 100 ms) of the pre-steady-state phase (Figure 3 and Table III). For both Mg^{2+} and Mn^{2+} the steady-state rate that is ultimately achieved is, within experimental error, that expected on the basis of coupled enzyme assays.

In the presence of α-anomer levels lower than FBPase subunit concentrations but total [Fru-1,6-P_2] > [FBPase], the amount of P_i liberated with Mg^{2+} is halved; i.e., one P_i per FBPase is rapidly formed within 5 ms and a second equivalent of P_i during the remainder of the pre steady state. Thus the pre-steady-state behavior responds to the limitation imposed by insufficient α anomer—bearing in mind that total Fru-1,6-P_2 is greater than FBPase and at a level still sufficient to totally populate all active sites (binding α and β forms).

One may question whether the spontaneous anomerization of β → α is sufficiently rapid to supply the 4 μM α-Fru-1,6-P_2 turned over by the second subunit. When a β-Fru-1,6-P_2 pool of 16 μM is assumed, the calculated rate of anomerization is 23 μM/s (1.45 s^{-1} × 16 μM β-Fru-1,6-P_2) (Midelfort et al., 1976), sufficient to furnish 2.3 μM in the 100-ms span of the pre steady state. This calculation presumes that both free and bound β-Fru-1,6-P_2 may anomerize and employs a rate constant from the original NMR experiments obtained in the absence of Mg^{2+} or Mn^{2+}. For comparison, the rate of pre-steady-state phase (Mg^{2+}) is 80 μM/s and does not respond to the limiting α-anomer concentration. Granting the nature of the experiments, it is not possible to conclude as to whether the enzyme in this conformational state catalyzes a significant mutarotation (β → α) process at this subunit or utilizes the β species directly.

Chase experiments conducted during the steady-state turnover indicate that there is no tightly bound Fru-1,6-P_2 at any of the active sites with Mg^{2+} as the catalyst; i.e., Δ = 0 (Table II). This is consistent with the liberation of 4 equiv of P_i/FBPase at excess α-anomer levels and the absence of hydrolyzable Fru-1,6-P_2 at any of the four active sites during steady-state turnover. However, pre-steady-state chase experiments further underscore the unusual nature of one subunit in the initial FBPase conformational state. In the presence of limiting α anomer where only two subunits turn over in the pre steady state, the remaining Fru-1,6-P_2 (1 equiv) at the time of the chase does not dissociate from the enzyme.

For Mn^{2+}, limiting α-Fru-1,6-P_2 levels do not affect the amount of P_i liberated since at 32 μM FBPase subunits and 8 μM α anomer one P_i per subunit is released within 5 ms (Figure 4). The extrapolation to 21 μM (rather than 16 μM) is misleading, since the steady-state rate is not that expected (93 vs. 34 μM/s) (Table III). Thus extrapolation of the corrected rate yields an intercept equivalent to the turnover of an additional 1 equiv of P_i/FBPase. Although earlier studies (Frey et al., 1977) gave identical intercept data, we had interpreted them in terms of rapid utilization of the total α-anomer pool rather than the turnover of two subunits because of the limitation imposed on the subsequent steady-state rates by the β → α anomerization. However, it is now clear by further restriction of the total Fru-1,6-P_2 pool that two subunits of the enzyme are active in the pre-steady-state phase regardless of α-anomer levels. Furthermore, the rate of the pre-steady-state phase (180 μM/s) is insensitive to anomer levels. Thus, the same β → α calculation and reasoning apply as given above for Mg^{2+}.

The lack of α-anomer availability is manifest in the steady-state phase, whose rate is 3-fold below that expected. On the presumption that the available β-Fru-1,6-P_2 pool at 100 ms is 25 μM, the first-order β → α anomerization rate constant is 1.3 s^{-1} [34 μM s^{-1} (25 μM)$^{-1}$] under these conditions, in good agreement with the NMR measurements. The rate limitation is not imposed by depletion of the Fru-1,6-P_2 pool since up to 75% of the substrate was hydrolyzed in other experiments without the corresponding retarding effect.[4]

In contrast to Mg^{2+}, in chase experiments with Mn^{2+} approximately 1.5 equiv of Fru-1,6-P_2 is chased/FBPase to Fru-6-P and P_i in the steady state. This is in accord with the less than stoichiometric release of P_i (two P_i per four FBPase subunits) noted above as well as the negligible dissociation of Fru-1,6-P_2 from the ternary FBPase·Fru-1,6-P_2·Mn^{2+} complex (Caperelli et al., 1978). The near total occupancy of the four subunits agrees with our calculations that Fru-1,6-P_2 is saturating. Thus with Mn^{2+}-dependent catalysis bound Fru-1,6-P_2 does not dissociate from the steady-state conformational species of FBPase before its conversion to P_i and Fru-6-P. Chase experiments at pre-steady-state times following the turnover of one subunit at limiting α-anomer concentrations reveal, as noted for Mg^{2+}, the presence of a nondissociating equivalent of Fru-1,6-P_2 per FBPase.

[4] A similar analysis is not possible with Mg^{2+} as the catalyst owing to the similarity between the rate constant for anomerization (1.5 s^{-1}) and k_{cat} (0.8 s^{-1}).

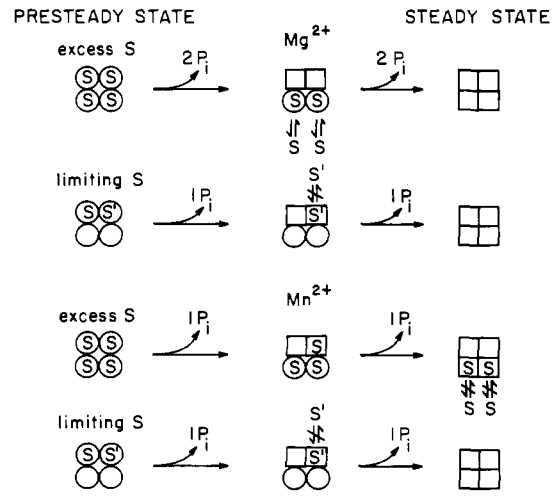

FIGURE 5: Minimal kinetic sequence required by rapid-quench and pulse–chase experiments for FBPase where S and S′ are α- and β-Fru-1,6-P_2, respectively. (○) or (□) designates FBPase subunits in the pre-steady-state and steady-state conformations, respectively. The presence of a bound Mn^{2+} per subunit as well as Mn^{2+} or Mg^{2+} complexed to S is not indicated. In the case of limiting S, the data do not reveal whether two of the subunits are occupied by S′.

In summary, the data support a catalytic cycle for the enzyme whose rate is not limited by the chemical step of P–O bond cleavage. Moreover there is no obvious phosphoryl enzyme as is found for many phosphatases (Bell & Koshland, 1971). Depending on the nature of the metal ion and the Fru-1,6-P_2/FBPase ratio, the pre-steady-state phase results in the release of 2 or 4 equiv (Mg^{2+}) of P_i or 2 equiv (Mn^{2+}) of P_i per FBPase. The release of 2 equiv of P_i in the presence of the Mn^{2+} parallels the binding of 2 equiv of the α and β anomers of methyl D-fructofuranoside 1,6-bisphosphate per FBPase in the presence of Mn^{2+} (Benkovic et al., 1978a). One interpretation of the change in pre-steady-state P_i stoichiometry is that only half the sites need turn over for the tetramer to adopt a second active conformational form (Fersht, 1977). The nature of the metal ion also dictates the rate of Fru-1,6-P_2 dissociation, suggesting that a model more complex than two conformational states is required to accommodate all our observations. The minimal kinetic sequence required by our data is shown in Figure 5 with utilization of the β anomer (S′) directly, or after anomerization by FBPase, in the pre steady state, furnishing a unique means of coping with low Fru-1,6-P_2 levels. It also appears possible to bypass the pre-steady-state cycle by incubating the products of the hydrolysis, Fru-6-P and P_i, with the enzyme.[5]

The internal equilibrium in the case of Mg^{2+} is displaced greatly in favor of products—no Fru-1,6-P_2 is detected in the chase experiments. For Mn^{2+} the position of the equilibrium is equivocal: either (1) two of the subunits possess only unreacted Fru-1,6-P_2 whereas the other two have present only P_i and Fru-6-P or (2) all four subunits have an internal equilibrium ≈ 1. By analogy to Mg^{2+}, the former interpretation is preferred. In contrast to the kinases (Nageswara Rao et al., 1979), which also catalyze an exergonic reaction ($K = 10^2$ for the Fru-1,6-$P_2 \rightleftharpoons$ Fru-6-P + P_i equilibrium, pH 6.8) (Lawson & Veech, 1979), there is no displacement of the internal equilibrium toward unity and no associated catalytic advantage to the enzyme (Albery & Knowles, 1976). It would appear that FBPase in the steady-state cycle exists primarily in the form of E·P_i·Fru-6-P and E·Fru-6-P complexes unless the activity of water (usually assumed to be unity) has been dramatically increased at the active site to compensate for the lack of measurable bound Fru-1,6-P_2. This would mean that the substrate·FBPase complex should be viewed as E·Fru-1,6-P_2·H_2O rather than E·Fru-1,6-P_2 (H_2O = 1) with H_2O not in equilibrium with bulk solvent. However, the shift in apparent internal equilibrium away from unity is not a general property of phosphatases since the internal equilibrium of myosin (ATP $\rightleftharpoons$ ADP + P_i at pH 8) is 9 (Trentham, 1977). Thus a second mode may have evolved for hydrolysis of phosphate and possibly other esters.

[5] A preliminary experiment showed that enzyme–Mn^{2+} exposed to unlabeled Fru-1,6-P_2 (product not removed) did not show an initial fast turnover in a reaction with ^{32}P-labeled substrate.

References

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 459.

Bell, R. M., & Koshland, D. E. (1971) *Science (Washington, D.C.) 172*, 1253.

Benkovic, P. A., Bullard, W. P., de Maine, M. M., Fishbein, R., Schray, K. J., Steffens, J. J., & Benkovic, S. J. (1974) *J. Biol. Chem. 249*, 930.

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

Benkovic, P. A., Caperelli, C. A., DeMaine, M., & Benkovic, S. J. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2185.

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

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

Benkovic, S. J., Engle, J. L., & Mildvan, A. S. (1972) *Biochem. Biophys. Res. Commun. 47*, 852.

Benkovic, S. J., Frey, W. A., Libby, C. B., & Villafranca, J. J. (1974) *Biochem. Biophys. Res. Commun. 57*, 196.

Caperelli, C. A., Frey, W. A., & Benkovic, S. J. (1978) *Biochemistry 17*, 1699.

de Maine, M. M., & Benkovic, S. J. (1972) *Arch. Biochem. Biophys. 152*, 272.

de Maine, M. M., & Benkovic, S. J. (1979) *Biochem. Biophys. Res. Commun. 88*, 835.

Dudman, N. P. B., de Maine, M. M., & Benkovic, S. J. (1978) *J. Biol. Chem. 253*, 5712.

Fersht, A. (1977) *Enzyme Structure and Mechanism*, p 215, W. H. Freeman, San Francisco.

Frey, W. A., Fishbein, R., de Maine, M. M., & Benkovic, S. J. (1977) *Biochemistry 16*, 2479.

Jencks, W. P., & Gilchrist, M. (1964) *J. Am. Chem. Soc. 86*, 1410.

Lawson, J. W. R., & Veech, R. L. (1979) *J. Biol. Chem. 254*, 6528.

Libby, C. B., Frey, W. A., Villafranca, J. J., & Benkovic, S. J. (1975) *J. Biol. Chem. 250*, 7564.

Martin, J. B., & Doty, D. M. (1949) *Anal. Chem. 21*, 965.

Midelfort, C. F., Gupta, R. K., & Rose, I. A. (1976) *Biochemistry 15*, 2178.

Nageswara Rao, B. D., Kayne, F. J., & Cohn, M. (1979) *J. Biol. Chem. 254*, 2689.

Nimmo, H. B., & Tipton, K. F. (1975) *Biochem. J. 145*, 323.

Rose, I. A. (1980) *Methods Enzymol. 64*, 47.

Traniello, S., Pontremoli, S., Tashima, Y., & Horecker, B. L. (1971) *Arch. Biochem. Biophys. 146*, 161.

Trentham, D. R. (1977) *Biochem. Soc. Trans. 5*, 5.

Ulm, E. H., Pogell, B. M., de Maine, M. M., Libby, C. B., & Benkovic, S. J. (1975) *Methods Enzymol. 42*, 369.

Wilkinson, K. D., & Rose, I. A. (1979) *J. Biol. Chem. 254*, 12567.