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Investigation of the Pre-Steady-State Kinetics of Fructose Bisphosphatase by Employment of an Indicator Method†

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ABSTRACT: The pre-steady-state kinetics for the hydrolysis of fructose 1,6-bisphosphate by rabbit liver fructose bisphosphatase have been investigated by stopped-flow kinetics utilizing an acid–base indicator method that permits the continuous monitoring of the inorganic phosphate product. The reaction sequence is characterized by two successive first-order steps followed by establishment of the steady-state rate. The first exponential process results from a conformational change in the protein that is dye sensitive owing to a perturbation of an acidic residue on the protein. A second process reflects the rapid initial turnover of all four subunits of the enzyme with the concomitant release of inorganic phosphate followed by the rate-limiting step of the catalytic cycle. This latter step may involve a product release (fructose 6-phosphate) or a second conformational change. The catalytic cycle ends with decay of the enzyme to its initial unreactive resting state.

In an effort to elucidate additional mechanistic details of the hydrolysis reaction catalyzed by fructose bisphosphatase (FBPase),[1] our interest centered on the locus of the rate-determining step(s) as well as the possibility of covalently bound intermediates. An investigation of the pre-steady-state kinetics offered the potential of some insight into these questions. Initial experiments monitoring fructose-6-P formation with a coupled enzyme assay system that produces NADPH indicated that the lag phase inherent in this method interfered with the observation of the pre-steady-state kinetics. It appeared possible, however, to exploit the pH change arising from the hydrolysis of fructose-1,6-P_2 in order to develop a continuous assay based on a dye indicator couple for inorganic phosphate that would be responsive on a millisecond time scale. This paper describes the resulting method and findings relevant to the mechanism of action of FBPase.

Experimental Procedures

Materials

Fructose bisphosphatase (rabbit liver) was purified by the method of Ulm et al. (1975) as modified by Benkovic et al. (1974). Glucose-6-P dehydrogenase (yeast) and phosphoglucose isomerase (yeast) were obtained as crystalline suspensions in ammonium sulfate from Sigma. Tetrasodium fru-1,6-P_2, disodium fru-6-P, NADP, tetrasodium EDTA, and Tris were purchased from Sigma. Tris was recrystallized from 95% ethanol–water containing 0.0001% EDTA prior to use. The substrate analogue (α+β)-methyl D-fructofuranoside-1,6-P_2 was prepared as previously described (Benkovic et al., 1970). Phenol red (Fisher) was purified according to the method of Freas & Provine (1928). All other chemicals including $MgCl_2 \cdot 4H_2O$ were reagent grade. Double-distilled, deionized, and degassed water was used throughout.

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[1] Abbreviations used: FBPase, fru-1,6-P_2, fru-6-P, and P_i are used throughout this paper to symbolize fructose bisphosphatase, fructose 1,6-bisphosphate, fructose 6-phosphate, and inorganic phosphate, respectively.

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Apparatus

Stopped-flow experiments were carried out with a Durrum D-110 system; pH measurements and titrations were performed using a Radiometer TTTlc titrator equipped with a SBU1a syringe buret and PHA 630T scale expander. Titrations were carried out under nitrogen in a cell thermostated at 25 °C. Ultraviolet spectra were recorded on a Cary 118 spectrophotometer equipped with a thermostated cell compartment. Enzyme assays were performed with either a Cary 118 or Gilford 240 spectrophotometer.

Methods

Determination of Buffer Capacities. The general procedure for the measurement of the buffer capacity of the solution components employed their rapid titration under nitrogen from pH 6.0 to 8.5 by the addition of 0.0197 N KOH from a calibrated microburet. pH readings were taken at 0.01-mL intervals. Solutions titrated had the following compositions: (1) 1.03 mM fructose-1,6-P_2, 5 mM $MgCl_2$, and 0.1 M KCl; (2) 1.0 mM fructose-6-P, 1.0 mM P_i, 5 mM $MgCl_2$, and 0.1 M KCl; (3) 1.4 mM phenol red, 5 mM $MgCl_2$, and 0.1 M KCl; (4) 1.0 mM EDTA, 5 mM $MgCl_2$, and 0.1 M KCl; (5) 49.5 μM FBPase, 5 mM $MgCl_2$, 10 μM EDTA, and 0.1 M KCl; (6) 49.5 μM FBPase, 5 mM $MgCl_2$, 10 μM EDTA, 0.1 M KCl, and 50 μM phenol red; and (7) 37.4 μM FBPase, 4 mM $MgCl_2$, 8 μM EDTA, 0.1 M KCl, and 156 μM (α+β)-methyl D-fructofuranoside-1,6-P_2. Duplicate titrations were done on solutions 6 and 7.

Determination of OH^-/Fru-1,6-P_2 Conversion Factor. Measurement of the hydroxide equivalents liberated during the hydrolysis of fru-1,6-P_2 was carried out in a series of pH-stat experiments. Degassed solutions, 0.2–0.8 mM in fructose-1,6-P_2 containing 0.1 M KCl, 5 mM $MgCl_2$, and 10 μM EDTA, were adjusted to pH 7.50 ± 0.02 in an autotitrator cell maintained under nitrogen. Aliquots (5 μL) of FBPase (ca. 0.05 mg) that had been preincubated in 5 mM $MgCl_2$, 10 mM Tris (pH 7.5) were added to initiate the hydrolysis. The pH was maintained within a ±0.05 tolerance by incremental addition of 1 mM HCl in 0.1 M KCl using the autotitrator. At t_∞ the hydroxide equivalents were calculated from the titrant consumed. The concentration of fructose-6-P at t_∞ was determined by coupled enzyme assay as a check on the initial fru-1,6-P_2 concentration and the completeness of the hydrolysis. With this conversion factor, the specific activity of FBPase was calculated from the initial rate of titrant consumption.

Fructose Bisphosphatase Assay. The specific activity of FBPase at 25 °C was determined by monitoring the rate of change at 340 nm of NADPH concentration in a solution containing 5 mM $MgCl_2$, 10 μM EDTA, 0.2 mM fru-1,6-P_2 (added last after a 15 min preincubation of FBPase with other components), 0.2 mM $NADP^+$, and 50 mM Tris (pH 7.5) with excess phosphoglucose isomerase and glucose-6-P dehydrogenase. The specific activity of FBPase ranged from 3.9 to 4.2 units/mg. The specific activity of FBPase was similarly measured in the presence of 0–150 μM phenol red and 0.1 M KCl to match stopped-flow conditions. The titer of fru-1,6-P_2 and fru-6-P solutions was determined by this procedure employing ϵ_{340} = 6220 M^{-1} cm^{-1} for the NADPH.

Determination of ΔA^{555} for Fru-1,6-P_2 Hydrolysis in the Presence of Phenol Red. Solutions (2 mL) 0.025–0.8 mM in fru-1,6-P_2, 5 mM $MgCl_2$, 10 μM EDTA, 18 μM phenol red, and 0.1 M KCl were adjusted to pH 7.50 ± 0.05 with 0.1 N KOH in the autotitration cell under nitrogen. One aliquot was removed by syringe and injected into a septum capped cuvette (previously purged with nitrogen), and a background A^{555} (absorbance at 555 nm) was established. A solution (1 μL) of FBPase (ca. 0.01 mg), preincubated in a solution of 5 mM $MgCl_2$, 10 μM EDTA, at pH 7.5 (Tris) for 10 min under nitrogen was then added to the solution in the autotitrator cell. Aliquots were removed at various times until a constant A^{555} at t_∞ was recorded. Identical experiments were also carried out with solutions 35 and 48 μM in phenol red.

pK_a of Phenol Red. The A^{555} of 20 solutions ranging from 1 to 16 μM in phenol red, pH 10 (5 mM borate, 0.1 M KCl), yielded a value of $\epsilon_{BH} = 5.69 \times 10^5$. The A^{555} of 20 phenol red solutions 0.1 M in HCl yielded ϵ_B = 600. Spectrophotometric titration of phenol red in the presence of 5 mM $MgCl_2$ and 0.1 M KCl by the method of Albert & Sargent (1962) gave a $pK_a = 7.785 \pm 0.019$.

Stopped-Flow Studies. Three reaction solutions, the first containing 5 mM $MgCl_2$, 0.1 M KCl, 10 μM EDTA, 38–44 μM phenol red, and varying (0.18–0.27 mM) fru-1,6-P_2 or (0.2 mM) (α+β)-methyl D-fructofuranoside-1,6-P_2, the second, 5 mM $MgCl_2$, 10 μM EDTA, 0.1 M KCl, 38–44 μM phenol red, and 12–18 μM FBPase, and the third, 0.1 M KCl, 0.2–0.3 mM Tris (pH 7.6), 5 mM $MgCl_2$, 10 μM EDTA, and 38–44 μM phenol red, were prepared in an autotitrator all under positive N_2 flow and adjusted to pH 7.60 ± 0.05. The solutions were then stored in separate reservoir syringes that were compatible with the stopped-flow system. The following protocol was then followed. A base line for the experiment was established by loading solution 3 into one of the two drive syringes and repeating measurements until a constant A_3^{555} was established. From A_3^{555} the pH of solution 3 then was calculated from the relationship

$$pH = pK_a + \log [(\epsilon - \epsilon_{BH})/(\epsilon_B - \epsilon)]$$

where $\epsilon = A^{555}/$[phenol red] and the remaining terms have the values reported above. Solution 2 containing enzyme was then introduced into the second drive syringe and repeatedly mixed with solution 3 by activating the stopped-flow system until A_{2+3}^{555} was constant. The absorbance of the enzyme solution was calculated directly from $A_2^{555} = 2A_{2+3}^{555} - A_3^{555}$ (where the subscripts identify the solution) owing to the fact that the buffer capacities of solutions 2 and 3 match. The pH of solution 2 was then calculated from the above relationship between pK_a and ϵ. Solution 3 in the drive syringe was replaced by 1 and the latter's pH established from A^{555} as above for solution 3. This procedure served as a check on the pH values set initially within the autotitrator cell. If the pH values for solutions 1 and 2 were not within pH 7.60 ± 0.05, the offending solution was readjusted. A series of stopped-flow experiments were then carried out with the pH values of solutions 1 and 2 being checked midway and at the end of the experimental series.

Computer Programs. Simulations of the OH^- vs. time reaction progress curves were performed using an interactive chemical reaction simulator program, written in FORTRAN by Dr. W. E. Brugger. The algorithm used (Shindell & Magagnose, 1976) involves calculation of the reaction time course by means of Taylor series expansions of the differential equations describing the changes in concentration of the chemical species in the system. The data from the stopped-flow experiments (changes of A^{555}) were transformed first to pH and then to relative OH^- concentration by a simple interactive FORTRAN program (see Appendix).

Results

Proton Uptake Calculations. The pH change anticipated upon hydrolysis of fru-1,6-P_2 in the pH region 6–8 can be readily calculated from the reaction cycle shown in Scheme

Scheme I

$$\begin{array}{ccc} ROPO_3H^- + H_2O & \longrightarrow & ROH + H_2PO_4^- \\ \Big\| \Big\downarrow K_{a_1} & & \Big\| \Big\downarrow K_{a_2} \\ ROPO_3^{2-} + H_2O & \longrightarrow & ROH + HPO_4^{2-} \end{array}$$

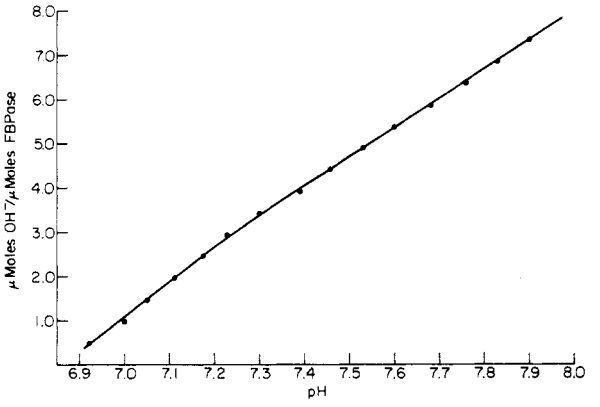

FIGURE 1: Titration of FBPase at 25 °C, μ = 0.1 in the presence of 5 mM $MgCl_2$ and 10 μM EDTA.

I where K_{a_1} is the dissociation constant for the 1-phosphate of fru-1,6-P_2 and K_{a_2} is the second dissociation constant for H_3PO_4. The quantity of protons (n_{H^+}) required to maintain constant pH over the course of the hydrolysis assuming constant volume is given by eq 1, where a_H is the activity of

$$n_{H^+} = -[\text{fru-1,6-P}_2]\frac{a_H}{K_{a_1} + a_H}\left(\frac{K_{a_2} - K_{a_1}}{K_{a_2} + a_H}\right) \qquad (1)$$

hydrogen ion as measured by the glass electrode. It is assumed that the ionization state of the 6-phosphate does not change as a result of the reaction. Since $K_{a_2} < K_{a_1}$, pH is increasing during the hydrolysis. Although in principle it is possible to calculate from eq 1 the conversion factor at any pH ($\delta_{OH}/\delta_{\text{fru-1,6-P}_2}$) that relates the moles of hydroxide ion formed per mole of fru-1,6-P_2 hydrolyzed, a direct determination is necessary owing to the difficulty in assigning discrete pK_a values to the 1-phosphate moiety (McGilvery, 1965). The average value ($\delta_{OH}/\delta_{\text{fru-1,6-P}_2}$) determined from pH-stat titrations is 0.113 ± 0.011 at pH 7.50. The specific activity of FBPase determined by this method agrees satisfactorily (within ±10%) with that obtained via coupled enzyme assay.

Measurements Employing Phenol Red. Preliminary to the stopped-flow experiments, the relationship between changes at A^{555} (free base form of phenol red) and variations in [fru-1,6-P_2] was established. The following factors were taken into consideration: choice of experimental pH; buffer capacity or proton uptake by all reaction components including FBPase; choice of indicator; possible interaction of the indicator dye with the enzyme; and buffer capacity of the medium. The choice of the experimental pH compromised a maximum in the value of n_{H^+} (midway between K_{a_1} and K_{a_2}) with lower buffer capacities for the reaction components and enzyme. Moreover, since the method is based on measuring ΔpH over the course of the reaction, a pH region was favored where the FBPase activity is pH insensitive. The pH region chosen was 7.50 ± 0.20. The buffer capacities (δ_{OH}/δ_{pH}) of the various solution components were determined by titrating solutions of fru-1,6-P_2, fru-6-P, phenol red, P_i, and EDTA in the presence of $MgCl_2$. The choice of phenol red as the dye indicator was based on its favorable pK_a (7.78) ensuring a linear response to changes in pH and its high ϵ_B (5.69×10^5 M^{-1} cm^{-1}). Titration of the enzyme is illustrated in Figure

Table I: Buffer Capacity Values Determined by pH Titration

components[a]	buffer capacity (δ_{OH}/δ_{pH})[b]
fru-1,6-P_2	0.195
fru-6-P + P_i	0.46
phenol red	6.87
EDTA	0.58
FBPase	0.066

[a] In the presence of 5 mM $MgCl_2$ and 0.1 M KCl. [b] Per unit concentration of component, pH 7.5 (25 °C).

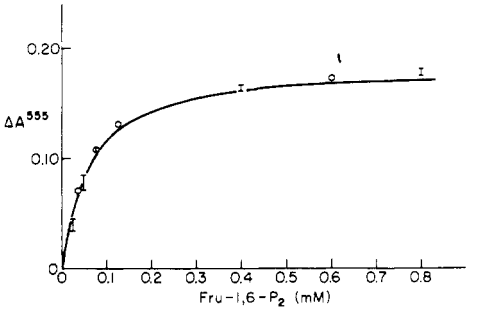

FIGURE 2: Change in A^{555} of phenol red as a function of [fru-1,6-P_2] hydrolyzed. Calculation of the solid line is described in the Appendix; conditions are defined in the Experimental Procedures.

1. The buffer capacity of the enzyme is not altered in the presence of the competitive inhibitor (α+β)-methyl D-fructofuranoside-1,6-P_2, under conditions where 2 mol of analogue is bound per mol of enzyme (Benkovic et al., 1978). Values of (δ_{OH}/δ_{pH}) employed in subsequent calculations are listed in Table I. Proton uptake by the medium was negligible under these conditions owing to the operational neutral pH and the use of degassed, deionized water. Insignificant interaction of the phenol red with FBPase was indicated by no measurable change in the steady-state assay at phenol red concentrations up to 50 μM. Moreover an equilibrium binding dialysis experiment at 20 μM phenol red revealed no significant dye binding to the enzyme.

Two tests of the accuracy of the buffer capacity and conversion factor measurements were undertaken, as well as a check on whether all influences on the observed A^{555} had been considered. In the first, the initial rate of fru-1,6-P_2 hydrolysis catalyzed by FBPase at pH 7.5 was determined both by coupled enzyme assay and by coupling to phenol red. The relationship between the two assays is given by eq 2, where

$$\frac{d(pH)}{dt} = -\frac{d[\text{fru-1,6-P}_2]}{dt}\left(\frac{\delta_{OH}}{\delta_{\text{fru-1,6-P}_2}}\right)\Big/\sum_N^N\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_N n_N \qquad (2)$$

n_N is the number of μmol of the Nth component. Since this is an initial rate assay, the contribution of fru-6-P and P_i to the total buffer capacity has been neglected. The comparison between the observed and predicted in terms of A^{555} gives dA^{555}/dt = 0.012 (calcd 0.015) at 0.6 mM fru-1,6-P_2 and dA^{555}/dt = 0.023 (calcd 0.024) at 0.4 mM fru-1,6-P_2.

A more stringent test includes the effect on proton uptake of the products, fru-6-P and P_i. In this case solutions of varying [fru-1,6-P_2] were totally hydrolyzed in the presence of phenol red and catalytic concentrations of FBPase and the ΔA^{555} recorded. A plot of ΔA^{555} vs. [fru-1,6-P_2] is shown in Figure 2 with the solid line calculated as described in the Appendix. In addition the concentration of phenol red was varied at 75 μM fru-1,6-P_2. At 35 μM phenol red, ΔA^{555} = 0.172 (calcd 0.168); at 48 μM phenol red, ΔA^{555} = 0.219 (calcd 0.200). The excellent agreement between the calculated and observed

Table II: Initial Proton Release and Fru-1,6-P_2 Hydrolysis on Reaction with FBPase[a]

[FBPase][b]	[fru-1,6-P_2][b]	pH mix[c]	pH min[d]	ΔOH obsd[e]	ΔOH calcd[f]	[H^+]/[FBPase][g]	no. of runs
6	180	7.55 ± 0.04	7.51 ± 0.03	2.6 ± 0.3	2.6	0.865 ± 0.16	7
6	180	7.44 ± 0.02	7.39 ± 0.02	2.8 ± 0.3	2.6	0.786 ± 0.17	5
6	250	7.59 ± 0.03	7.52 ± 0.03	3.3 ± 0.4	2.6	1.36 ± 0.48	15
9	270	7.54 ± 0.04	7.48 ± 0.04	3.3 ± 0.4	4.0	0.907 ± 0.24	5

[a] Determined in stopped-flow spectrophotometer at 25 °C, [phenol red] = 38 μM. [b] In μM. [c] Initial pH upon mixing. [d] pH drop observed after mixing. [e] "Burst height" estimated by extrapolation to t_{min}. [f] As described in Results, expressed in μM, equivalent to 1.0, 1.1, 1.3, and 0.8 [fru-1,6-P_2]/[FBPase]. [g] ($[H^+]_{mix} - [H^+]_{min}$)/[FBPase].

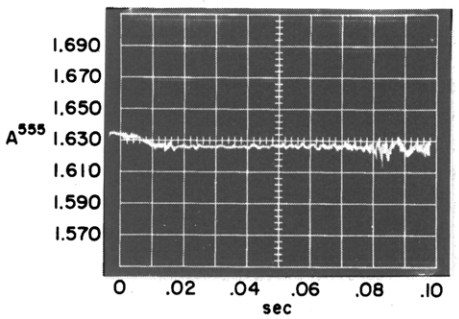

FIGURE 3: Stopped-flow trace of A^{555} vs. time obtained in a control experiment upon mixing FBPase, EDTA, $MgCl_2$, and phenol red in one syringe with EDTA, $MgCl_2$, and phenol red in the second syringe. Concentrations are given in the Experimental Procedures.

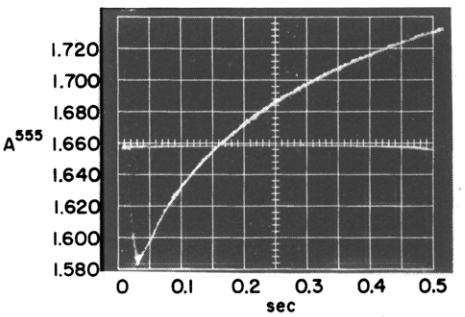

FIGURE 4: Stopped-flow trace of A^{555} vs. time upon mixing FBPase (6 μM), EDTA, $MgCl_2$, and phenol red in one syringe with fru-1,6-P_2 (250 μM), EDTA, $MgCl_2$, and phenol red in the second syringe. Concentrations of other components are listed in the Experimental Procedures.

values for ΔA^{555} indicates that the experimental values and computations of buffering capacity are correct.

The plot of Figure 2 also establishes the concentration range for fru-1,6-P_2 to be employed in the stopped-flow experiments. The ΔA^{555} is insensitive to [fru-1,6-P_2] at concentrations greater than 400 μM because this component and its hydrolysis products principally determine the buffer capacity of the solution. The finding that the buffer capacity of a solution of FBPase and (α+β)-methyl D-fructofuranoside-1,6-P_2 (at concentrations where at least two subunits are saturated) is simply the sum of the individual buffer capacities of the components further supports its employment in those cases where the concentration of the enzyme–substrate complex(es) is the major fraction of the total enzyme concentration.

Stopped-Flow Experiments. The initial mixing experiments with one syringe containing FBPase, EDTA, $MgCl_2$, and phenol red and the second syringe containing EDTA, $MgCl_2$, and phenol red, both at pH 7.50, 25 °C, and 0.1 M in KCl, gave no indication of proton uptake or release (Figure 3). Inclusion of fru-1,6-P_2 produced the changes in A^{555} as a function of time depicted for a typical experiment in Figure 4. In general A^{555} decreased exponentially at times less than 20 ms, followed by a delayed exponential increase at times up to 250 ms, and then increased linearly.

It is necessary to first transform the data from A^{555} to pH as indicated in the Appendix. Since the change in total buffer capacity during hydrolysis (up to 400 ms) is only 5–6% at 180–270 μM fru-1,6-P_2 as only 29 and 25% of the available fru-1,6-P_2 is hydrolyzed, the total buffer capacity would be closely approximated by the summation of buffer capacities arising from the initial solution components. The result of such a transformation is shown in Figure 5 and as expected owing to the linear relationships between A^{555} and pH the principal features observed in the A^{555} vs. time trace (Figure 4) remain.

The two first-order exponential processes are associated with characteristic amplitudes which are listed in Table II. The initial rapid decay of A^{555} results in the average release of 1.1 ● 0.3 [H^+]/[FBPase] or 0.25 [H^+] per mol of subunit. The

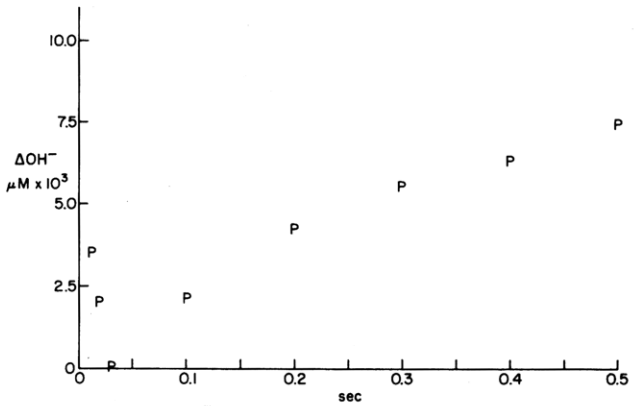

FIGURE 5: Transformation of "A^{555} vs. time" data given in Figure 4 by the method in the Appendix to "ΔOH vs. time".

transient encountered during hydrolysis of the substrate has an average amplitude in ΔOH of 3.0 compared with 2.6 (calcd) at 6 μM FBPase and 3.3 compared with 4.0 (calcd) at 9 μM FBPase. The computation of ΔOH was carried out as described in the Appendix determining BC_i at the midpoint of a segment equal to $n_{FBPase}/n_{fru\text{-}1,6\text{-}P_2}$ (the fraction of fru-1,6-P_2 hydrolyzed per turnover). This calculation assumes all four subunits are catalytically active in the initial turnover (vide infra). Alternatively the amplitude can be expressed in terms of [fru-1,6-P_2]/[FBPase] ≃ 1.1 ± 0.2 (per active site).

The rate coefficients for the first-order rate processes can be determined directly from the trace of A^{555} vs. time by graphical analyses and do not depend on total buffer capacity (Fersht, 1977). A more accurate fitting procedure involved computer simulation of the transformed data (ΔOH vs. t) in order to generate the required rate coefficients as well as the linear steady-state rate (k_{cat}). Fitting of the results by this procedure is shown in Figure 6 with values listed in Table III. It was necessary to include a first-order rate constant between the two observed first-order exponential processes in order to account for the delay in the A^{555} increase after the initial rapid

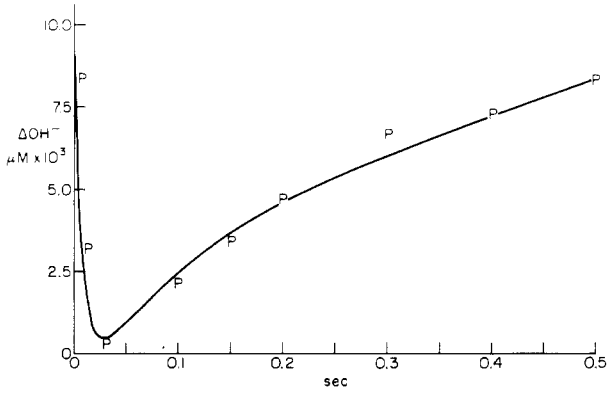

FIGURE 6: Computer simulation of the ΔOH vs. time data for FBPase (9 μM) and fru-1,6-P_2 (270 μM) according to the kinetic model given in Scheme II. Values for the rate coefficient employed are listed in Table III.

Table III: Rate Coefficients for the First-Order and Zero-Order Rates Observed for FBPase + Fru-1,6-P_2 in the Stopped-Flow Indicator Method

FBPase[a]	fru-1,6-P_2[a]	k_1[b]	k_1'[b,c]	k_2[b]	k_3[b]
6	180	140	(30)	14	4
6	250	140	(30)	14	4
9	270	150	(30)	13	3

[a] In μM. [b] For the steps designated in Scheme II. [c] Estimated from the duration of the delay in A^{555} increase.

Scheme II

$$E + \text{fru-1,6-}P_2 \overset{K_S}{\rightleftharpoons} E\cdot\text{fru-1,6-}P_2 \overset{k_1}{\longrightarrow} \overset{k_1'}{\longrightarrow} E'\cdot\text{fru-1,6-}P_2 \quad \text{(i)}$$

$$E'\cdot\text{fru-1,6-}P_2 \overset{k_2}{\longrightarrow} E'' + P_i \quad \text{(ii)}$$

$$E'' \overset{k_3}{\longrightarrow} E'; \; E' + \text{fru-1,6-}P_2 \overset{K_S}{\rightleftharpoons} E'\cdot\text{fru-1,6-}P_2 \quad \text{(iii)}$$

$$E' \overset{\text{fast}}{\longrightarrow} E \quad \text{(iv)}$$

proton release. Failure to do so resulted in a simulated curve that was not compatible with the experimental data. Combination of (α+β)-methyl D-fructofuranoside-1,6-P_2 with FBPase also leads to a rapid exponential decrease in A^{555} with a similar first-order rate coefficient (107 s^{-1}) but with approximately 30% the amplitude observed with fru-1,6-P_2. Both first-order rate coefficients (k_1 and k_2) do not exhibit a dependency on fru-1,6-P_2 concentration at these substrate levels.

Discussion

The minimal mechanism describing the FBPase reaction, at pH 7.5, with the rate coefficients determined in this study is given in Scheme II. The steps of Scheme II will be discussed in numerical order.

The initial first-order decrease in pH observed after mixing FBPase with either fru-1,6-P_2 or the competitive inhibitor (α+β)-methyl D-fructofuranoside-1,6-P_2 (de Maine & Benkovic, 1972) is attributed to a sequence involving rapid ligand binding followed by stepwise conformational changes. One of the latter causes an alteration in the environment of an acidic residue, lowering its pK_a and resulting in net proton release. The specific activity of FBPase measured at pH 7.5 depends on the pH of the incubation medium for the enzyme and shows a transition between two activity states with a pK_a ≃ 7.64 (Dudman et al., 1978). Presuming that the same residue is involved in the events of step i, a $\Delta pK_a \simeq 0.2$ unit occurs in this residue per subunit after substrate binding. The value of 1 μM assigned K_s is an average value based on previous binding experiments involving substrate (Libby et al., 1975), substrate analogues (Benkovic et al., 1978) and steady-state kinetic measurements (Dudman et al., 1978). Under the present conditions the fru-1,6-P_2 is at saturating concentrations, with respect to the reactive α anomer (20% of fru-1,6-P_2; Frey et al., 1977). Alternatively step i might involve the sequence

$$E \overset{k_1}{\rightleftharpoons} E' + \text{fru-1,6-}P_2 \overset{K_s}{\rightleftharpoons} \overset{k_1'}{\longrightarrow} E'\cdot\text{fru-1,6-}P_2$$

with an initial slow conformational change, although at the high substrate levels employed this sequence might give rise to a first-order process dependent on fru-1,6-P_2 concentration (Fersht, 1977).

The second step involves the rapid initial turnover of all four subunits of the enzyme as implicated by the amplitude of the first-order increase in pH or hydroxide concentration which translates to 1.2 fru-1,6-P_2 turnovers per subunit. The finding that all subunits are catalytically active in the initial turnover suggests that FBPase has a significantly greater affinity for the α anomer by a minimal factor of 20-fold, confirming a conclusion reached in earlier isotope-trapping experiments (Caperelli et al., 1978) despite the fact that β-substrate analogues act as competitive inhibitors with equivalent K_I values (de Maine & Benkovic, 1972). Consequently there is no evidence for half-site reactivity in this system.

Step ii depicts the release of P_i from the product complex that is detected by the acid–base indicator dye. It is less likely that the formation of a covalent phosphoryl-FBPase intermediate or a noncovalent P_i·FBPase would be dye sensitive unless either event was associated with proton uptake by a basic residue(s) on the enzyme. The rapid formation of P_i (in the case of Mn^{2+}, $k_2 > 50$ s^{-1}) has been noted in quench experiments that would release acid-labile phosphate from the enzyme (Frey et al., 1977). In addition the amplitude of the first-order process approximates the enzyme concentration which is consistent with two consecutive, irreversible (owing to P_i loss) first-order processes (Gutfreund, 1972) where the magnitude of the burst is given by $(k_2/[k_2 + k_3])^2 \simeq 0.7$ in the 9 μM FBPase experiments. The fact that the average experimentally observed amplitude is greater than a 1:1 stoichiometry for 6 μM FBPase might be caused by a systematic error in one of the component concentrations, in particular that of the enzyme which contributes up to 40% of the overall buffer capacity. Consequently computer simulations of those runs where the amplitude is greater than 1.0 for [fru-1,6-P_2]/[FBPase] do not accurately reflect the experimental amplitude; however, the rate coefficients for the two first-order processes can be accurately obtained.

The step in which fru-6-P is released has not been established. Both fru-6-P and P_i act as competitive inhibitors toward FBPase employing Mn^{2+} as the catalytic metal ion (Dudman et al., 1978). In equilibrium binding experiments both fru-6-P and P_i bind to FBPase in the presence of Mn^{2+} (Benkovic et al., 1978). In the presence of Mg^{2+} the affinity of FBPase for fru-6-P is slightly reduced, but that for P_i becomes negligible (Benkovic et al., 1978). Consequently step iii may involve release of fru-6-P in a product-limiting rate-determining step that principally determines the k_{cat} for the enzyme. From steady-state assays the average specific activity of FBPase ≃ 2–3 units/mg, equivalent to k_{cat} = 4–6 s^{-1}. From the stopped-flow data, $k_{cat} = k_3k_2/(k_2 + k_3) \simeq 2$–3 s^{-1}. Alternatively step iii may involve a second slower conformational transition not associated with product release. In the presence of excess substrate, the catalytic loop involving

steps ii and iii is maintained, eventually decaying to yield the resting state of the enzyme.

The sensitivity of the phenol red method—the total change in pH in the runs reported is 0.06–0.07 pH unit—has suggested its application to other problems which involve phosphoryl transfer, particularly those with ATP (Bagshaw & Trentham, 1974; Chock & Eisenberg, 1974). The rapidity of the proton-transfer processes that equilibrate the indicator dye with changes in substrate concentration ensure that catalytic processes involving the enzyme that occur on the stopped-flow time scale will limit the rate of the indicator–dye response. A dye–indicator method has been previously employed to measure the pK_a of an enzyme–substrate complex formed with chymotrypsin (Fersht & Renard, 1974), an experiment that also should be possible with FBPase owing to the H^+ release observed upon binding of fru-1,6-P_2. The finding that the resting and active states of the enzyme are not the same offers a rationale for the variation in the efficacy of various analogues as inhibitors depending on whether their addition is to cycling or resting enzyme (Frey et al., 1977).

Appendix

The total change in A^{555} was calculated by dividing the course of the hydrolysis into five equal segments and determining the total buffer capacity in a volume element of 1 mL at the midpoint of each segment. The buffer capacity of the ith segment is given by

$$BC_i = \sum^{N}\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_N = \sum^{5} n_{\text{fru-1,6-P}_2}\left[(0.2i - 0.1)\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_{\text{fru-6-P}} + (1.1 - 0.2i)\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_{\text{fru-1,6-P}_2}\right] + \sum^{N-2}\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_{N-2} n_{N-2}$$

where n is the quantity of a given solution component in μmol and N is the total number of components. The quantity of OH^- formed in each segment is

$$\delta_{OH} = 0.2 n_{\text{fru-1,6-P}_2}\left(\frac{\delta_{OH}}{\delta_{\text{fru-1,6-P}_2}}\right)$$

Since

$$\frac{\delta_{OH}}{\delta_{BC_i}} = \delta_{\Delta pH_i}$$

the total change in pH is given by

$$\Delta pH = \sum^{5} \Delta pH_i$$

and the total change in A^{555} by

$$\Delta A^{555} = C \sum^{5} \Delta pH_i$$

where

$$C = 2.303[\text{phenol red}]\epsilon_B\left(\frac{K_a a_H}{(K_a + a_H)^2}\right)$$

The transformation of $(\Delta A^{555}/\Delta t)$ obtained in the stopped-flow experiments to $(\Delta OH/\Delta t)$ was accomplished by dividing the reaction time into segments of 50 μs. The ΔpH_i for the ith time segment was calculated from

$$\frac{\Delta pH_i}{\Delta t} = \log\left(\frac{\epsilon_i - \epsilon_{BH^+}}{\epsilon_B - \epsilon_i}\right)\left(\frac{\epsilon_B - \epsilon_{i-1}}{\epsilon_{i-1} - \epsilon_{BH^+}}\right)$$

where $i - 1 = 0$ at pH_{min}. The buffer capacity of the ith time segment was determined from

$$BC_i = n_{\text{fru-1,6-P}_2}\left(1 - \frac{i_N}{\sum^{N} i_N}\right)\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_{\text{fru-1,6-P}_2} + n_{\text{fru-1,6-P}_2}\left(\frac{i_N}{\sum^{N} i_N}\right)\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_{\text{fru-6-P}} + BC_0$$

where N is the number of time segments and

$$BC_0 = \sum^{N-2}\left(\frac{\delta_{OH}}{\delta_{pH}}\right)_{n-2} n_{N-2}$$

where n and N have the above definitions. It follows that

$$\left(\frac{\Delta OH_i}{\Delta t}\right) = \left(\frac{\Delta pH_i}{\Delta t}\right)(BC_i)$$

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