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Isotope-Trapping Experiments with Rabbit Liver Fructose Bisphosphatase†

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ABSTRACT: Isotope-trapping experiments with metal-free rabbit liver fructose 1,6-bisphosphatase have shown that enzyme-bound D-fructose 1,6-bisphosphate completely dissociates prior to enzyme turnover initiated by Mn^{2+} as the catalytic metal. The exchange rate of the binary enzyme-D-fructose 1,6-bisphosphate complex with the substrate pool is, therefore, more rapid than its conversion to products, suggesting that structural Mn^{2+} is necessary for productive substrate binding. Rapid-quench isotope-trapping experiments confirm the requirement for structural Mn^{2+} ions for productive binding to occur. These experiments also show that an ordered formation of the enzyme-$Mn^{2+}{}_s$-D-fructose 1,6-bisphosphate ternary complex which features metal-ion addition prior to substrate constitutes a catalytically competent pathway in the mechanism of fructose 1,6-bisphosphatase and that all four subunits are active in a single turnover event.

Fructose 1,6-bisphosphatase (EC 3.1.3.11, D-fructose 1,6-bisphosphate 1-phosphohydrolase, FBPase[1]) catalyzes the hydrolysis of D-fructose 1,6-bisphosphate (fru-1,6-P_2) to D-fructose 6-phosphate (fru-6-P) and orthophosphate (P_i) in the presence of a required divalent metal cation cofactor. Neutral rabbit liver FBPase[2] has been shown to bind four Mn^{2+} ions per mole of enzyme tetramer. An additional second set of four Mn^{2+} ions is also bound to the enzyme in the presence of the substrate analogue ($\alpha + \beta$) methyl D-fructofuranoside 1,6-diphosphate (Libby et al., 1975). For purposes of discussion, the first set of Mn^{2+} ions has been designated structural Mn^{2+} ($Mn^{2+}{}_s$) and the second set as catalytic Mn^{2+} ($Mn^{2+}{}_c$). The existence of this second set of binding sites in the presence of

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[1] The abbreviations FBPase, fru-1,6-P_2, fru-6-P and P_i are used throughout this paper to symbolize fructose 1,6-bisphosphatase, D-fructose 1,6-bisphosphate, D-fructose 6-phosphate, and ortho phosphate, respectively; other abbreviations used are: NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP, EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] Neutral enzyme exhibits optimal activity at pH 7.5 in the presence of known activators, in contrast to the proteolytically modified alkaline form of the enzyme, which exhibits maximal activity at alkaline pH.

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substrate has also been reported for the alkaline form of the enzyme (Grazi et al., 1971). The metal-free neutral enzyme is also known to bind four molecules of fru-1,6-P_2 per mole of tetramer (Libby et al., 1975).

Studies of the steady-state kinetics of FBPase-catalyzed hydrolysis of fru-1,6-P_2 in the presence of Mn^{2+} ions indicated that the mechanism could be described by either a random equilibrium scheme or by a rapid equilibrium or steady-state ordered scheme (Dudman and Benkovic, 1977). Binding data, which revealed only one rather than two sets of metal-ion binding sites in the absence of a substrate analogue favored an ordered scheme featuring binding of substrate to the enzyme-$Mn^{2+}{}_s$ complex, followed by activation by the catalytic metal ion (Libby et al., 1975).

In order to evaluate the catalytic competency of the binary FBPase-fru-1,6-P_2 and ternary $Mn^{2+}{}_s$-FBPase-fru-1,6-P_2 complexes, a series of experiments employing the isotope-trapping (pulse-chase) method was conducted. In this technique, which was pioneered by Meister and Rose and their co-workers (Krishnaswamy et al., 1962; Rose et al., 1974), enzyme and radiolabeled substrate are incubated, followed by the simultaneous addition of a large excess of unlabeled substrate and any other substrates or cofactors necessary for enzymatic activity. The formation of labeled product under one-turnover conditions establishes the catalytic competency of the initial enzyme-substrate complex or more precisely the partitioning of the complex to product rather than its dissociation to free substrate. The present study employs this technique with FBPase, with and without structural Mn^{2+} ions, incubated with [1-^{32}P]fru-1,6-P_2, followed by the simultaneous addition of excess unlabeled substrate and Mn^{2+} ions. Determination of the amount (if any) of labeled phosphate released as product as a function of metal-ion concentration yields information concerning the rate of dissociation of the binary complexes (with and without $Mn^{2+}{}_s$) and, at infinite concentrations of metal ion, the rate of dissociation of the ternary complex to the respective binary complex vs. its rate of conversion to product. These results also bear directly on the validity of the ordered kinetic sequence as a description of a particular catalytic pathway (Fromm, 1976).

Experimental Procedures

Materials

Fru-1,6-P_2, fru-6-P, $NADP^+$, EDTA, glucose-6-phosphate isomerase, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Chelex-100 was obtained from Bio-Rad. Tris buffers were prepared from Trizma base (Sigma, reagent grade) which had been recrystallized from 95% ethanol containing 0.0001% EDTA. Hepes buffers were prepared from Hepes (Sigma, reagent grade). The ampholine electrofocusing apparatus and carrier ampholytes were purchased from LKB Instruments. Instabray scintillation cocktail was obtained from Yorktown Research. Inorganic salts were reagent grade. Young, fasted, rabbit livers were purchased from Pel-Freez Biologicals.

Carrier-free $H_3{}^{32}PO_4$ (10 mCi), obtained from New England Nuclear, was used with commercially available enzymes and substrates to prepare [γ-^{32}P]ATP by the method of Keenan et al. (1972). Purified [γ-^{32}P]ATP and fru-6-P were reacted with phosphofructokinase to yield [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 Benkovic et al. (1974b). Disc and sodium dodecyl sulfate gel electrophoresis were performed as described previously (Benkovic et al., 1974b), with one sharp protein band observed for the purified enzyme. Protein concentrations were determined on the basis of the standard absorbance of 0.71 for a 1.0 mg/mL solution. Enzyme for the enzyme-substrate pulse-chase experiments was further purified by isoelectric focusing over the pH range 3.5–10 and by column chromatography on Chelex-100 equilibrated in 50 mM Tris-HCl (pH 7.5).

The enzyme was assayed spectrophotometrically by following the rate of NADPH production at 340 nm in the presence of excess glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase. The routine assay solution for activity at 25 °C contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $NADP^+$, FBPase, 7.6 units of glucose-6-phosphate 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.

Extraction Analysis for $^{32}P_i$. Inorganic phosphate was separated from sugar phosphates by an adaptation of the Jencks and Gilchrist modification (1964) of the Martin and Doty procedure (1949). Assay tubes contained 0.6 mL of an isobutyl alcohol–benzene (water saturated) solution and 0.6 mL of a solution made from 2 g of ammonium molybdate, 5.55 mL of concentrated H_2SO_4, and water added to make 100 mL. A known volume of the quenched reaction solution was added to the assay tube and the contents were vigorously agitated for two separate 30-s intervals. Low-speed centrifugation produced clean separation of the two layers. Equal volumes of each layer were added to scintillation vials containing 10 mL of Instabray scintillation cocktail and ^{32}P activity was measured with a Beckman LS 255 liquid scintillation spectrometer. Comparison of the radioactivity in the organic phase to that in the aqueous phase gave the relative distribution of ^{32}P as P_i and acid-stable organic phosphate. Corrections were made for variations in the volumes of the two layers due to the volume of the reaction solution added to the two-phase assay system.

Pulse-Chase Experiments. Metal-free FBPase (0.5 μM) was incubated with [1-^{32}P]fru-1,6-P_2 (0.5–2.0 μM) in a 0.25-mL pulse solution containing 50 mM Tris-HCl (pH 7.5) for 1 min at room temperature. The concentration of the binary enzyme subunit-fru-1,6-P_2 complex varied from 0.4 to 0.8 μM based on the stoichiometric binding constants determined previously (Libby et al., 1975). The Mn^{2+}-fru-1,6-P_2 chase solution (0.05 mL) was then injected into the vigorously stirred pulse solution, resulting in a solution containing 0.05–0.5 mM $MnCl_2$ and 5.0 mM fru-1,6-P_2. Varying the stirring rate did not alter the observed results, indicating that the rate of isotope dilution is not a factor. Aliquots were removed at 10 and 45 s and 2, 5, 10, and 20 min, and the reaction was quenched by the addition of 0.3 mL of 10% $HClO_4$. The extent of reaction at each time was determined from the ratio of $^{32}P_i$ to [1-^{32}P]-fru-1,6-P_2 via the extraction assay described above. The converse experiments were performed in which the enzyme was incubated with unlabeled fru-1,6-P_2 and [1-^{32}P]fru-1,6-P_2 was added in the chase solution. The same study was also performed employing Mg^{2+} and Zn^{2+} as catalytic metal ions (as the chloride and nitrate salts, respectively).

Rapid-Quench Experiments. The rapid-quench kinetic experiments were performed utilizing a Durrum Multi-Mixer apparatus as modified in this laboratory (Benkovic et al., 1974a). Controls for the pulse-chase experiments were performed by mixing equal volumes of enzyme-$MnCl_2$ and [1-^{32}P]fru-1,6-P_2-$MnCl_2$ for times varying from 70 to 370 ms,

followed by quenching with 2 volumes of 5% $HClO_4$. The reaction solutions for each time point contained 50 mM Hepes (pH 7.5), 0.5 μM FBPase, 15 μM [1-^{32}P]fru-1,6-P_2, and 6.5 μM $MnCl_2$. The extent of reaction at each time point was determined by measuring the ratio of $^{32}P_i$ to [1-^{32}P]fru-1,6-P_2 via the extraction assay outlined above. The reaction time points were varied randomly to guard against system-generated errors.

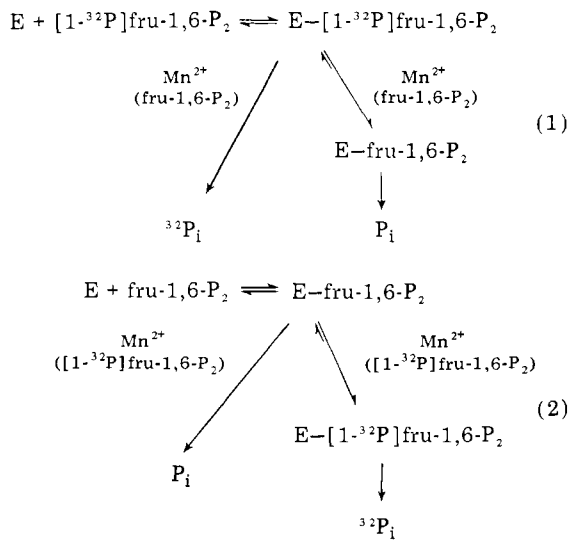
The pulse-chase rapid-quench kinetics were performed by mixing equal volumes of enzyme-$MnCl_2$ and [1-^{32}P]fru-1,6-P_2-$MnCl_2$, with the mixing time held at 70 ms, and then chasing with 2 volumes of unlabeled fru-1,6-P_2-$MnCl_2$, followed by quenching at varying times with 1 volume of 10% $HClO_4$. The distribution of $Mn^{2+}{}_s$-FBPase-[1-^{32}P]fru-1,6-P_2 complexes at 70 ms was identical to those described above in the control experiments. The reaction solutions at each quench time point contained 50 mM Hepes (pH 7.5), 0.25 μM FBPase, 7.5 μM [1-^{32}P]fru-1,6-P_2, 1 mM fru-1,6-P_2, and 18–43 μM $MnCl_2$. The free Mn^{2+} concentrations in the reaction solution ranged from 5.7 to 13.6 μM after correction for complexation with fru-1,6-P_2 (Dudman and Benkovic, 1977).

Calculation of [1-^{32}P]fru-1,6-P_2 Trapped. The $^{32}P_i$ cpm extrapolated to zero time from the rapid-quench pulse-chase experiments were corrected by subtraction of the $^{32}P_i$ cpm contributed by the background rate. The net $^{32}P_i$ cpm represent the amount of [1-^{32}P]fru-1,6-P_2 hydrolyzed in one turnover, hence, the amount of [1-^{32}P]fru-1,6-P_2 trapped. The $^{32}P_i$ cpm were converted to μM [1-^{32}P]fru-1,6-P_2 hydrolyzed via the relationship $^{32}P_i$ cpm/sp act. of [1-^{32}P]fru-1,6-P_2.

Results

Pulse-Chase Experiments. Scheme I represents the design of the pulse-chase experiment. Equation 1 (Scheme I) describes the isotope-trapping experiment, in which tetrameric enzyme was incubated with from 1 to 4 molar excess of [1-^{32}P]fru-1,6-P_2. This solution was then chased with a solution containing Mn^{2+} ions and unlabeled fru-1,6-P_2, resulting in a 2500- to 10 000-fold excess of fru-1,6-P_2/[1-^{32}P]fru-1,6-P_2 and permitting convenient monitoring of the hydrolysis reaction without recourse to rapid quenching. For the converse experiments outlined in eq 2 (Scheme I), FBPase was pulsed with unlabeled fru-1,6-P_2, followed by chasing with [1-^{32}P]-fru-1,6-P_2 and Mn^{2+} ions, at concentrations identical to those

SCHEME I



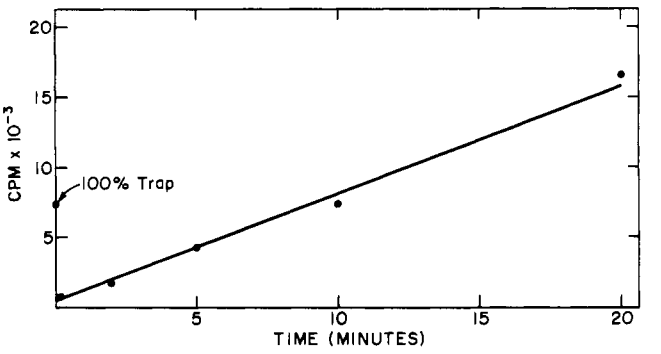


FIGURE 1: Pulse-chase (enzyme-substrate) experiments with 0.5 μM FBPase and 2 μM [1-^{32}P]fru-1,6-P_2 in the pulse solution. The chase resulted in a final Mn^{2+} ion concentration of 0.05 mM. The data are plotted as $^{32}P_i$ cpm released with time. The arrow represents the $^{32}P_i$ cpm expected if the enzyme-substrate complex had been totally trapped.

used in the isotope-trapping experiment. Therefore, the concentration of the enzyme-fru-1,6-P_2 binary complex is identical in both sets of experiments. In each case, the extent of reaction at each time point was determined from the amount of $^{32}P_i$ formed and the amount of unreacted [1-^{32}P]fru-1,6-P_2.

The results of a typical experiment are shown in Figure 1. The initial rate of production of $^{32}P_i$ is linear and extrapolates to zero $^{32}P_i$ (within experimental error) at zero time. Extrapolation to zero time measures the amount of fru-1,6-P_2 converted to product from the initial binary complex, i.e., one turnover, and circumvents the problem of substrate inhibition resulting from the high unlabeled fru-1,6-P_2 concentration of the chase solution if the experiments were limited to a constant time interval. This problem is manifest in the evaluation of the partitioning rate constants from the pulse-chase data which should be determined under conditions where the enzyme rate is maximal. The arrow represents the $^{32}P_i$ cpm expected for complete trapping of the binary enzyme-[1-^{32}P]fru-1,6-P_2 complex, its concentration being calculated from the stoichiometric binding constants determined earlier (Libby et al., 1975).

A comparison of the rates of $^{32}P_i$ production for both the isotope-trapping and converse experiments for two Mn^{2+} concentrations and also for Zn^{2+} and Mg^{2+} are shown in Figure 2. Neither experimental protocol suggests any trapping of the binary fru-1,6-P_2-FBPase complex has occurred at [Mn^{2+}] concentrations as high as 0.5 mM (equivalent to $[Mn^{2+}]_{free}$ = 0.42 mM). The data indicate that the observed release of $^{32}P_i$ over the 20-min reaction period is identical for both experiments. Furthermore, the required identity of rates for $^{32}P_i$ release in both experiments, the slopes of the plots shown in Figure 2, is only obtained by assuming that the enzyme-bound labeled substrate completely dissociates prior to turnover, resulting in complete mixing of its label with the substrate pool of the chase solution and a corresponding reduction in the specific radioactivity value used to compute the amount of P_i released. Although the rate of hydrolysis of [1-^{32}P]fru-1,6-P_2 differs for the various metal ions, the identity in rates of the pulse-chase and converse reaction calculated as above dictates the identical conclusion, namely, that the total dissociation of the binary enzyme-fru-1,6-P_2 complex occurs before its conversion to product.

Rapid-Quench Experiments. The design of the rapid-quench experiments is outlined in Scheme II. In these experiments, the pulse solution consists of 0.19 μM FBPase complexed with three $Mn^{2+}{}_s$ ions, 0.22 μM FBPase complexed with

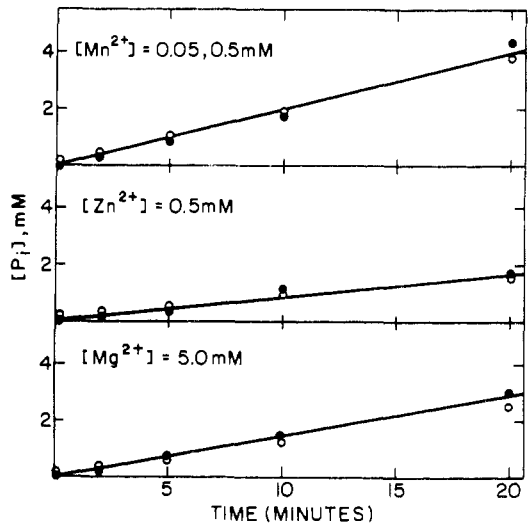
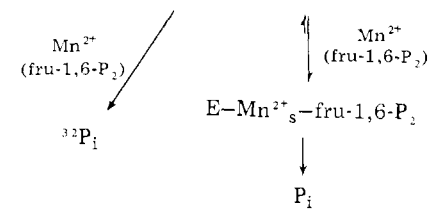


FIGURE 2: Pulse-chase (○) (0.5 μM FBPase and 2 μM [1-^{32}P]fru-1,6-P_2) and converse (●) (0.5 μM FBPase and 2 μM fru-1,6-P_2) experiments with Mn^{2+}, Zn^{2+}, or Mg^{2+} in the chase resulting in the metal ion (total) concentrations indicated.

SCHEME II



four Mn^{2+}_s ions, and 0.54 nM uncomplexed FBPase based on the stoichiometric binding constants previously determined (Libby et al., 1975). At these levels of Mn^{2+}, a high proportion of the FBPase contained at least three Mn^{2+}_s ions, yet only ~13% of the [1-^{32}P]fru-1,6-P_2 was hydrolyzed during the 70-ms incubation time which is an acceptable background correction. The substrate/enzyme ratio employed (30:1) was sufficient to ensure that the initial turnover was not limited by the availability of the reactive α-anomer of fru-1,6-P_2 which comprises ~20% of the total anomeric distribution (Frey et al., 1977) and furthermore that the substrate concentration would remain saturating (Libby et al., 1975). The solution was then chased with a solution containing Mn^{2+} and 1 mM fru-1,6-P_2, such that the final free Mn^{2+} concentration ranged from 5.7 to 13.6 μM, and unlabeled fru-1,6-P_2 was in a 130-fold excess.

As noted above, control quench experiments were employed to establish the background fru-1,6-P_2 hydrolysis during the 70-ms incubation interval in which a fraction of the reactive complex Mn^{2+}_s–FBPase–fru-1,6-P_2–Mn^{2+}_c was generated, since not all of the Mn^{2+} was bound as Mn^{2+}_s. At longer time intervals up to 400 ms, the continued rate of formation of $^{32}P_i$ was linear, suggesting that the 70-ms incubation interval was sufficient to form the catalytically reactive enzyme species. The rapid-quench pulse-chase experiments were conducted with varying amounts of Mn^{2+} ions in the chase solution in order to titrate the enzyme-Mn^{2+}_s-[1-^{32}P]fru-1,6-P_2 ternary complex. The results with a low Mn^{2+} chase are shown in Figure 3a. The data indicate that a mole fraction (15%) of the ternary complex has been trapped, resulting in a burst of $^{32}P_i$ at zero time. Increasing the amount of Mn^{2+} in the chase solution results in increased trapping of the ternary complex, as

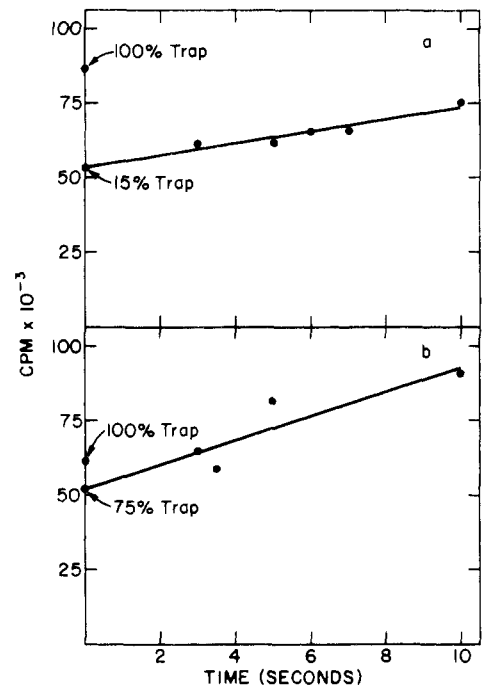


FIGURE 3: Rapid-quench experiments at 0.25 μM FBPase and 7.5 μM [1-^{32}P]fru-1,6-P_2. The chase resulted in a Mn^{2+} concentration of 15 μM (5.7 μM free Mn^{2+}) in a and a Mn^{2+} concentration of 25 μM (8.8 μM free Mn^{2+}) in b.

shown in Figure 3b, as well as an increased subsequent rate of turnover (greater slope) as anticipated.

The results for the entire series of Mn^{2+} ion concentrations are summarized in Table I. These data show that the enzyme-Mn^{2+}_s-[1-^{32}P]fru-1,6-P_2 ternary complex is capable of rapid generation of product upon the addition of catalytic Mn^{2+} ions, that the amount of trapping is a function of the catalytic Mn^{2+} ion concentration, and that stoichiometric trapping of the ternary complex can be achieved, i.e., one fru-1,6-P_2/subunit.

Discussion

Neutral FBPase possesses two kinds of sites for binding of Mn^{2+} to the tetrameric enzyme, four to which Mn^{2+} binds in the absence of substrate, referred to as "structural", and four sites that are detected only in the presence of substrate or substrate analogue termed "catalytic". In the absence of substrate, the stoichiometric binding constants for Mn^{2+} vary from $\sim 1 \times 10^6$ to 3.9×10^4 M^{-1}, owing to negative cooperativity (Libby et al., 1975). However, in the presence of a competitive substrate analogue such as (α + β) methyl D-fructofuranoside 1,6-diphosphate, no negative cooperativity is observed for the binding of the structural Mn^{2+} and, furthermore, their binding constants are increased by approximately an order of magnitude. The second "catalytic" set of Mn^{2+} which is observed to bind in the presence of analogue is characterized by a dissociation constant (calculated from the average stoichiometric binding constant) of 3.3×10^{-5} M which compares favorably with the K_m for Mn^{2+} of 1.5×10^{-5} M determined under saturating fru-1,6-P_2. Metal ions such as Mg^{2+} and Zn^{2+} can activate the enzyme for catalysis, although their division into "structural" and "catalytic" sites for the rabbit liver enzyme has not yet been finalized (Frey et al., 1976).

It has also been demonstrated that the metal-free enzyme binds fru-1,6-P_2 in a negatively cooperative fashion with the stoichiometric binding constants ranging from 2.9×10^7 to 4.8

TABLE I: Trap of [1-^{32}P]Fru-1,6-P_2 as a Function of Mn^{2+} Concentration.

$[Mn^{2+}]_{total}$ (μM)	$[Mn^{2+}]_{free}$ (μM)	[[1-^{32}P]Fru-1,6-P_2] (μM) trapped ($\pm\sigma$)	[1-^{32}P]Fru-1,6-P_2 trapped/subunit ($\pm\sigma$)
15	5.7	0.59 (0.23)	0.15 (0.06)
25	8.8	3.0 (0.83)	0.75 (0.20)
40	13.6	4.7 (0.54)	1.1 (0.13)

$\times 10^4$ M^{-1}. Consequently, it is of considerable interest to contrast the kinetic properties of the substrate–enzyme complex with and without $Mn^{2+}{}_s$ quantitatively in order to comment on the role of $Mn^{2+}{}_s$ in the catalytic process and to obtain further information characterizing the reaction sequence in the catalytic process as obtained with the isotope-trapping method. The results of the pulse-chase experiments with the binary enzyme–fru-1,6-P_2 complex (Figures 1 and 2) clearly show that all of the enzyme-bound substrate (up to 1.5 fru-1,6-P_2/FBPase) dissociates prior to turnover regardless of the identity of the chase metal ion (Mn^{2+}, Mg^{2+}, or Zn^{2+}) or its concentration. Two mechanistic interpretations pertaining to the overall enzymatic reaction are suggested by these results: (1) the reaction proceeds via an equilibrium mechanism where dissociation of the enzyme–fru-1,6-P_2 complex, and by inference an enzyme–fru-1,6-P_2–Mn^{2+} complex, is rapid relative to conversion to product or (2) the mechanism is ordered, such that structural metal must be bound prior to substrate in order for catalysis to be functional initially.

In sharp contrast are the results of the rapid-quench experiments (Table I) which show that at 40 μM $[Mn^{2+}]_{total}$ all of the fru-1,6-P_2 initially bound to the enzyme–$Mn^{2+}{}_s$ complex is converted to product without dissociation from the enzyme. Consequently, the ternary enzyme–$Mn^{2+}{}_s$–fru-1,6-P_2 complex is immediately functional. In addition, certain limits may be placed on the relevant rate constants designated according to Scheme III. From partition methods (Cleland, 1975):

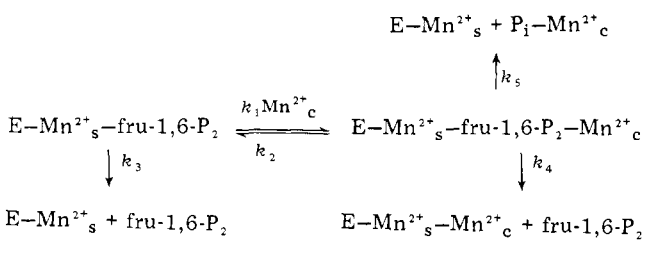
$$[E_T]\left(\frac{k_4}{k_5}\right) = \left(\frac{[E_T]}{[P_{i,max}]}\right)^{-1}$$

where $[P_{i,max}]$ is the maximum concentration of fru-1,6-P_2 trapped as P_i. Since $[E_T]/[P_{i,max}]$ approaches unity for $Mn^{2+}{}_{free} \simeq 13.6$ μM (correcting for binding to fru-1,6-P_2), $k_4 \ll k_5$ or dissociation of fru-1,6-P_2 from the intermediate quarternary complex is essentially suppressed. Although as written k_5 represents a component of product forming and release steps, one actually measures the rate of fru-1,6-P_2 dissociation relative to the first irreversible step leading to product. Equating $k_4 \simeq 0$, then the rate of dissociation of fru-1,6-P_2 from the initial ternary complex is given by:

$$k_3/V/[E_T] = K'/K_m{}^{Mn^{2+}}$$

where K' equals the Mn^{2+} concentration that results in the trapping of half the bound fru-1,6-P_2 trapped at infinite Mn^{2+}, and $K_m{}^{Mn^{2+}}$ is the Michaelis constant for Mn^{2+}. An upper limit of $Mn^{2+}{}_{free} \approx 7$ μM can be estimated for K', since a more accurate value cannot be obtained owing to dissociation of

SCHEME III



$Mn^{2+}{}_s$ from the enzyme at $[Mn^{2+}]_{total} \simeq 15$ μM. Under our conditions where all four enzyme active sites are saturated with fru-1,6-P_2, $K_m{}^{Mn^{2+}} \simeq 15$ μM and $V/[E_T] \simeq 50$–60 s^{-1} (Frey et al., 1977), so that k_3 is $\simeq 30$ s^{-1}. Owing to the fact that attempts to measure fru-1,6-P_2 formation in the reverse direction have been unsuccessful, one cannot presently conclude on the basis of this evidence alone that the k_3 dissociation step would be rate limiting in this direction or that the enzyme accumulates as the enzyme–$Mn^{2+}{}_s$–fru-1,6-P_2 complex.

Measurement of relative dissociation rates does not furnish evidence for a random as opposed to an ordered sequence of substrate and catalytic metal ion binding. However, the demonstration that the protocol employed in the rapid-quench experiments generates a catalytically functional complex directly supports an ordered sequence of fru-1,6-P_2 binding prior to $Mn^{2+}{}_c$ as a viable pathway. Moreover, owing to the slower release of fru-1,6-P_2 relative to its conversion to product, the sequence does not involve a rapid equilibrium binding of substrate. This observation is in accord with a similar conclusion reached from an examination of the steady-state kinetic patterns in which the alternative pathway involving catalytic Mn^{2+} binding prior to fru-1,6-P_2 did not appear operative at $[Mn^{2+}] \leq 1$ mM (Dudman and Benkovic, 1977).

In terms of an ordered sequence, the association rate predicted (Alberty and Hammes, 1958) for the first substrate (fru-1,6-P_2) is given by k_{cat}/K_m. For FBPase, $k_{on} = k_{cat}/K_m = 10^7$ M^{-1} s^{-1}, whereas k_{on} calculated from $k_3 \approx 30$ s^{-1} and $K_{dissoc} \approx 9.2 \times 10^{-7}$ M (Benkovic and Benkovic, 1977) gives $k_{on} \sim 3 \times 10^7$ M^{-1} s^{-1} in satisfactory agreement. It is apparent that the rate of combination of fru-1,6-P_2 with enzyme is considerably less than a diffusion-limited process and suggests that the k_{on} step leading to the first-formed reversible complex is a composite one, i.e., consisting of a diffusion-limited combination of enzyme and substrate, followed by a second, slower intracomplex process, perhaps involving a conformational change.

Experiments with less than four $Mn^{2+}{}_s$ ions bound per mole of enzyme were not attempted, since at intermediate Mn^{2+} concentrations there would be a statistical distribution of species. Although in principle the dissociation constant for fru-1,6-P_2 from the binary complex of enzyme–$Mn^{2+}{}_s$ also can be determined by this method, the experimental problems here owing to the low dissociation constant would be formidable. It is noteworthy, however, that at saturating concentrations of substrate, all the bound fru-1,6-P_2 evidently is hydrolyzed in the first turnover. Since the enzyme is α-anomer specific but binds the β-anomer nonproductively (Frey et al., 1977), this finding suggests that FBPase has a significantly greater affinity for the α-anomer by a minimal factor of 15- to 20-fold based on our experimental precision.

In conclusion, it appears that the role of $Mn^{2+}{}_s$ is to key the active site for maximum catalytic efficiency. In its absence, the sugar phosphate substrate rapidly dissociates from the enzyme, a process which can be completely suppressed by $Mn^{2+}{}_s$. The inability of $Mn^{2+}{}_s$ to rapidly bind in the presence of substrate may be simply a physical consequence of a steric factor imposed by the former's presence at the $Mn^{2+}{}_s$ binding site. It is also noteworthy that the use of the quench time delay

in the pulse-chase method, permitting an extrapolation to zero time to obtain a single turnover event, allows one to cancel out kinetic effects arising from the presence of excess cold substrate in the chase media. For the case of FBPase, these effects are a serious hazard owing to its inhibition by substrate at 1 mM concentrations.

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Fluorescent Phosphonate Label for Serine Hydrolases, Pyrenebutyl Methylphosphonofluoridate: Reaction with Acetylcholinesterase†

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ABSTRACT: Pyrenebutyl methylphosphonofluoridate reacts with acetylcholinesterase to yield a conjugate with a stoichiometry of one fluorescent label per 80 000 molecular weight subunit. Kinetics of inactivation by the phosphonate and the subsequent reactivation behavior, as well as competition between the fluorescent compound and diisopropyl fluorophosphate, indicate that equivalent active site serines are involved in the labeling reaction. The long wavelength absorption band of the pyrene conjugate exhibits an 8-nm bathochromic shift, whereas its fluorescence emission spectrum shows little change. The shift correlates best with wavelength maxima observed for pyrenebutanol in solvents of high refractive index and is indicative of an association between the pyrene moiety and aromatic residues in the active center of the enzyme. Dynamic fluorescence quenching of the conjugated pyrene moiety by iodide, nitromethane, and thallous ion is markedly reduced when compared with the corresponding quenching constants obtained for pyrenebutanol in solution. Quenching by thallous ion is most efficient and iodide is least efficient when quenching efficiencies for the conjugated fluorophore are considered relative to the free fluorophore. The absorption spectrum of the pyrene moiety overlaps with the emission spectrum of the enzyme tryptophanyl residues and phosphonylation results in a quenching of the tryptophan fluorescence. Propidium, a peripheral site ligand, binds with equal affinity to the phosphonylated and native enzyme. Propidium binding results in 85–90% quenching of pyrene fluorescence. These findings show pyrenebutyl methylphosphonofluoridate to be a specific active site fluorescent label which can serve as a donor and acceptor of fluorescence energy. The conjugated pyrene appears to be buried in a region of high polarizability where it is restricted in contact with solvent and where the dominant electrostatic effect on pyrene would suggest an anionic subsite in the vicinity.

Catalysis by acetylcholinesterase (AChE[1]) shows essential mechanistic features similar to those observed for other serine hydrolases whose crystal structures have been elucidated. This group of enzymes is characterized by a charge relay system which enhances the nucleophilicity of the active site serine (Henderson et al., 1971) and an oxyanion hole which serves to stabilize a tetrahedral transition state and bind certain tetrahedral inhibitors (Robertus et al., 1972). The serine within the active center of AChE is capable of nucleophilic attack upon esters of carbon, sulfur, phosphorus, and boron, forming acyl-enzyme intermediates which differ in the subsequent rate at which they deacylate to give active enzyme and hydrolysis products. Acetylcholine, for example, reacts with AChE to form the acetyl-enzyme intermediate which readily reacts with

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[1] Abbreviations used: AChE, acetylcholinesterase; PBMPF, pyrenebutyl methylphosphonofluoridate; PBMP-AChE, pyrenebutyl methylphosphonoacetylcholinesterase; PBOH, pyrenebutanol; PBA, pyrenebutyric acid; DFP, diisopropyl phosphorofluoridate; 2-PAM, 2-pyridinealdoxime methiodide; BSA, bovine serum albumin.

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