Distances between Structural Metal Ion, Substrates, and Allosteric Modifier of Fructose Bisphosphatase†

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ABSTRACT: The binding of two paramagnetic probes within a subunit of fructose bisphosphatase, viz., Mn^{2+} at a structural site and a nitroxide spin-label at a sulfhydryl site, has permitted the measurement of NMR and electron paramagnetic resonance (EPR) relaxation rates to map the active and allosteric site topography. Distances from these loci to the phosphoryl of fructose 6-phosphate (Fru-6-P) and inorganic phosphate (P_i) and four nuclei of adenosine 5′-phosphate (AMP) (the phosphorus nucleus, H-8, H-2, and H-1′) were obtained. These measurements located the Mn^{2+} approximately equidistant from the two phosphoryl moieties of the product ligands Fru-6-P and P_i and in close proximity to the AMP. The adenosine moiety of the latter is oriented anti. Analysis of EPR data revealed that the nitroxide group is ~16 Å from the structural Mn^{2+} site. Calculation of the residence times for the hydrolysis reaction products suggests that their dissociation should not be rate limiting in the overall reaction cycle.

Fructose bisphosphatase (FBPase)[1] consists of four subunits, each with the demonstrated capacity to bind a divalent structural metal ion (Mn^{2+}_s or Zn^{2+}_s) in the absence of substrate or product ligands. An additional four catalytic metal ions (Mn^{2+}_c or Zn^{2+}_c) bind in the presence of fructose 1,6-bisphosphate (Fru-1,6-P_2) or fructose 6-phosphate (Fru-6-P) plus inorganic phosphate (P_i) (Libby et al., 1975; Pontremoli et al., 1968a, 1978; Kolb & Kolb, 1973; Benkovic et al., 1978a). Presumably Mg^{2+} acts similarly, although its behavior has been inferred from competition experiments (Benkovic et al., 1978a; Pontremoli et al., 1978). The association of the products, Fru-6-P and P_i, has been analyzed in terms of four negatively cooperative sites with the binding of Fru-6-P occurring in the absence of divalent metal ion, although the extent of its binding is increased in the order $Mg^{2+} < Zn^{2+} < Mn^{2+}$, whereas the binding of P_i shows a requirement for divalent metal ion with Mn^{2+} being more effective than Mg^{2+} (Benkovic et al., 1978b). Binding of the allosteric inhibitor, AMP, is markedly enhanced by the presence of Fru-1,6-P_2, resulting in a single AMP residue per subunit (Benkovic et al., 1978b; Sarngadharen et al., 1969; Pontremoli et al., 1968b). The enzyme also possesses one reactive sulfhydryl group (toward 5,5′-dithiobis(2-nitrobenzoic acid)) per subunit (M. M. deMaine and S. J. Benkovic, unpublished experiments).

The binding of Mn^{2+} suggests measurement of its effect as a paramagnetic ion on NMR relaxation rates in order to map the active and allosteric site topography. Distances from Mn^{2+} to the phosphoryl of Fru-6-P, P_i, and AMP and the H-8, H-2, and H-1′ of AMP can be conveniently mapped. Distances to the phosphoryl groups in Fru-1,6-P_2 are more difficult to obtain owing to substrate reactivity. Two of the four structural Mn^{2+} ions associate with FBPase with equilibrium binding constants >1 μM^{-1} so that the concentration of an FBPase–$2Mn^{2+}_s$ complex can be made stoichiometric with added Mn^{2+}. A second locus for the distance measurements is obtained by spin-labeling the sulfhydryl site with a nitroxide radical and observing the effect of Mn^{2+}_s on the intensity of the nitroxide EPR signal.

Experimental Procedures

Materials. FBPase (rabbit liver) was purified by the method of Ulm et al. (1975) as modified by Benkovic et al. (1974). Metal ions were removed from the enzyme by extensive dialysis against 50 mM Tris-HCl (pH 7.5) which had been treated with Chelex (Benkovic et al., 1978a). A maleimide spin-label (2,2,5,5-tetramethylpyrrolidinyl-1-oxy-3-maleimide) was the gift of Dr. Betty Gaffney. All reagents were reagent grade. Solutions were treated with Chelex to remove metal ion contaminants before the addition of the desired metal ion. Doubly distilled deionized water was used throughout.

The concentration of AMP was determined by measuring the absorbance at 259 nm, using an extinction of 15 400 M^{-1} cm^{-1}. The concentration of Fru-6-P was determined by measuring the absorbance at 340 nm of NADPH produced in a solution containing Fru-6-P, 5 mM $MgCl_2$, 0.01 mM EDTA, 0.2 mM $NADP^+$, and 50 mM Tris-HCl (pH 7.5) upon the addition of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The number of reactive sulfhydryl groups per FBPase molecule before and after reaction with the maleimide spin-label was determined by measuring the absorbance produced at 412 nm by reacting Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), ϵ = 13 600 M^{-1} cm^{-1} for the reduced form) with the native and spin-labeled FBPase.

Methods. Measurements of the ^{31}P relaxation rates at 40.5 MHz were made by Fourier transform NMR on a JEOL PS-100-FT spectrometer equipped with a Nicolet computer and at 81 MHz on a Brüker WP-200 spectrometer. The measurement of ^1H relaxation rates was made at 200 MHz on the Brüker spectrometer. Longitudinal relaxation times, T_1, were measured by using at 180°–τ–90° pulse sequence. Transverse relaxation rates, $1/T_2$, were obtained by measuring

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[1] Abbreviations used: FBPase, fructose bisphosphatase; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; P_i, inorganic phosphate; AMP, adenosine 5′-phosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized NADP; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.

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the line width at half-height in hertz, Δ, and applying the equation $1/T_2 = \pi\Delta$.

All experiments were performed at 20 ± 1 °C. A typical 1.5-mL sample contained 13.5 mM phosphate (Fru-6-P and/or P_i or AMP) in 45 mM Tris-HCl (pH 7.5) containing 10% D_2O. The deuterium served as a heteronuclear lock. The AMP samples also contained 0.9 mM Fru-6-P. For most experiments an enzyme solution containing two metal ions (Mn^{2+} or Mg^{2+}) per FBPase tetramer was added to give solutions 0.8 and 1.6 μM in FBPase. A series of experiments were also performed at 13.5 mM Fru-6-P and P_i at a constant FBPase concentration of 3.32 μM with successive additions of metal ion to give 1, 2, 3, and 4 ratios of metal ion to FBPase. Those samples were allowed to equilibrate for 1 h after each addition of metal ion. Since there was no change in the relaxation rate normalized for Mn^{2+} concentration, $1/fT_{1p}$, during the metal ion titration, these studies indicate that contributions to the relaxation rates due to binary Mn^{2+}–Fru-6-P or Mn^{2+}–P_i complexes were not a contributory factor. Thus the NMR measurements reflect FBPase–Mn^{2+}–substrate(s) complexes. This was verified by EPR experiments.

Longitudinal and transverse relaxation times for the H-8, H-2, and H-1′ of AMP were measured at 200 MHz on 1.5-mL samples containing 13.5 mM AMP, 45 mM Tris-HCl (pH 7.5), 0.9 mM Fru-6-P, and 99% D_2O. The concentration of $2Mn^{2+}{}_s$–enzyme was 1.1 or 2.2 μM.

FBPase was labeled with the maleimide spin-label by adding enzyme (final concentration of 2 μM) to a solution of the maleimide (10 μg/mL) in 50 mM Tris-HCl (pH 7.5). The solution was incubated at room temperature in the dark for 2 h, after which it was dialyzed extensively against the same buffer to remove unreacted maleimide. The FBPase solution was then concentrated by using a collodion bag apparatus.

EPR measurements were conducted with a Varian E-12 spectrometer operating at 9.1 GHz. Samples were placed in 1-mm quartz cells at 20 ± 1 °C. Spectra of Mn^{2+} bound to FBPase were obtained in solutions containing 105 μM FBPase and one Mn^{2+} per FBPase (40-μL sample volume). Spin-labeled samples (50 μL) were 34 μM labeled FBPase and 0–0.80 mM in $MnCl_2$.

Distance Measurements. The distances between the various binding sites of rabbit liver FBPase were determined by measuring the effect of enzyme-bound Mn^{2+} on the spin-lattice relaxation rates ($1/T_1$) of the ^1H of AMP and ^{31}P nuclei of P_i, AMP, and Fru-6-P. The paramagnetic contribution ($1/T_{1p}$) to the measured relaxation rate was calculated according to the equation

$$1/T_{1p} = 1/T_1(\mathrm{Mn}) - 1/T_1(\mathrm{Mg}) \qquad (1)$$

where $1/T_1(Mn)$ is the spin–lattice relaxation rate in the presence of Mn^{2+} and $1/T_1(Mg)$ is the relaxation rate in the presence of Mg^{2+}. The Mn^{2+}–nuclei distances were calculated by using a modified form of the Solomon–Bloembergen equation (Solomon & Bloembergen, 1956)

$$r\ (\text{Å}) = C\left[fT_{1p}\left(\frac{3\tau_c}{1+\omega_I{}^2\tau_c{}^2}\right)\right]^{1/6} \qquad (2)$$

where f is the mole fraction of enzyme-bound ligand, τ_c is the correlation time for the dipolar interaction, and ω_I is the nuclear Larmor precession frequency. In this equation C has a value of 812 for Mn^{2+}–^1H interactions and 601 for Mn^{2+}–^{31}P interactions.

The distance between the nitroxide spin-label and $Mn^{2+}{}_s$ was determined by measuring the diminution of the EPR signal of the spin-labeled enzyme upon the addition of Mn^{2+} (Leigh, 1970). The distance (r) was calculated from eq 3 (Leigh, 1970)

$$r^6 = \frac{g\beta\mu^2\tau_c}{\hbar C} \qquad (3)$$

where the constants g, β, and $\hbar$ have the usual meanings, and μ and τ_c are the magnetic moment and the electron-spin relaxation time of Mn^{2+}, respectively. The value of C was obtained from the relative amplitude of the EPR spectrum at saturating Mn^{2+} by using the graph presented by Leigh (1970).

Table I: Paramagnetic Contributions to ^{31}P Relaxation Rates of P_i, Fru-6-P, and AMP at 40.5 MHz and Mn^{2+}-Nuclei Distances in FBPase–Mn^{2+}–Ligand Complexes

ligand[a]	$[2Mn^{2+}{}_s$–FBPase$]^b$ (μM)	$1/fT_{1p}$ ($\times 10^{-5}$ s^{-1})	$1/fT_{2p}$ ($\times 10^{-5}$ s^{-1})	r^c (Å)
P_i	0.8	3.5	4.7	6.3 ± 0.5
	1.6	2.6	4.3	
Fru-6-P	0.8	3.3	4.0	6.3 ± 0.5
	1.6	2.6	3.1	
AMP	0.8	10.5		5.2 ± 0.4
	1.6	9.8		

[a] The concentration of each ligand was 13.5 mM. Other details are given in the text. [b] In terms of FBPase concentration. [c] The Mn^{2+}–^{31}P distances were calculated from eq 2 by using the average of the $1/fT_{1p}$ values and a τ_c of 1.8×10^{-9} s. The error limits for these distances were calculated by assuming a 25% deviation in both the correlation time and the value for $1/fT_{1p}$.

Results

Effect of Mn^{2+}–FBPase on ^{31}P Relaxation Rates of P_i, AMP, and Fru-6-P. Shown in Table I are the $1/fT_{1p}$ and $1/fT_{2p}$ values obtained for the various enzyme–$Mn^{2+}{}_s$–ligand complexes. The dissociation constants for P_i, Fru-6-P, and AMP for FBPase are all <1 mM, and thus the enzyme is fully saturated with these substrates at the concentrations used in these experiments (Benkovic et al., 1978a,b). Mn^{2+} was added to apoenzyme in the proportion of 2 mol of metal ion/enzyme tetramer in order to ensure that there would be no complications from Mn^{2+} in the bulk solution and to avoid conditions where there might be more than one Mn^{2+} per subunit.

In an attempt to determine the paramagnetic effect exerted by the second set of Mn^{2+} ions ($Mn^{2+}{}_c$) on the ^{31}P relaxation rates of P_i and Fru-6-P, we titrated the enzyme with Mn^{2+}. In this experiment, 3.3 μM apo-FBPase (13.2 μM subunit concentration) was titrated with $MnCl_2$ to a concentration of 13.3 μM in four equal increments. The Fru-6-P and P_i concentrations were 13.5 mM each. Unfortunately titration beyond the 1:1 ratio resulted in ^{31}P resonances that were too broad to easily observe. The results are shown in Table II. This experiment also enabled us to detect any change in the Mn^{2+}–^{31}P distance when both Fru-6-P and P_i were bound to the enzyme at the same time. Within experimental error, the $Mn^{2+}{}_s$–^{31}P distances to P_i and Fru-6-P seem to be lengthened slightly when they are simultaneously bound to the enzyme.

Effect of Mn^{2+}–FBPase on ^1H Relaxation Rates of AMP. The distances from the bound Mn^{2+} of FBPase to the H-8, H-2, and H-1′ of AMP were determined by measuring the effect on Mn^{2+}–FBPase on the spin–lattice relaxation rates of these protons at 200 MHz. The results are shown in Table III.

Determination of Correlation Times and Distances in Mn^{2+}–FBPase Complexes. The correlation time for the dipolar effect of Mn^{2+}–FBPase on the nuclei of P_i, Fru-6-P, and AMP was measured in several ways. The first method was to measure the peak to peak separation of one of the EPR

Table II: Paramagnetic Contribution to ^{31}P Relaxation Rates of P_i and Fru-6-P as a Function of Mn^{2+} Concentration in the FBPase-Mn^{2+}-Fru-6-P Complex

expt	ligand[a]	$[Mn^{2+}]$[b] (μM)	$1/fT_{1p}$ ($\times 10^{-3}$ s^{-1})	r[c] (Å)
I	P_i	3.3	1.3	7.3 ± 0.6
	Fru-6-P	3.3	1.9	6.9 ± 0.5
II	P_i	6.6	1.6	7.1 ± 0.5
	Fru-6-P	6.6	1.7	7.0 ± 0.5
III	P_i	9.9	1.6	7.1 ± 0.5
	Fru-6-P	9.9	1.7	7.0 ± 0.5
IV	P_i	13.3	1.7	7.0 ± 0.5
	Fru-6-P	13.3	1.7	7.0 ± 0.5

[a] The concentrations of P_i and Fru-6-P were 13.5 mM each. [b] In terms of [$2Mn^{2+}{}_s$-FBPase]. [c] The distances were calculated by using eq 2 and a correlation time of 1.8×10^{-9} s. The error limits were calculated by using a 25% deviation in both the correlation time and the value for $1/fT_{1p}$.

Table III: Paramagnetic Contributions to ^1H Relaxation Rates of AMP at 200 MHz by the FBPase-Mn^{2+} Complex

proton[a]	[$2Mn^{2+}{}_s$-enzyme][b] (μM)	$1/fT_{1p}$ ($\times 10^{-3}$ s^{-1})	$1/fT_{2p}$ ($\times 10^{-3}$ s^{-1})	r[c] (Å)
H-8	1.1	70	135	4.1 ± 0.4
	2.2	50	103	
H-2	1.1	2.6		6.8 ± 0.5
	2.2	2.9		
H-1′	1.1	1.4		7.3 ± 0.5
	2.2	2.2		

[a] The concentration of AMP was 13.5 mM. Other details are listed in the text. [b] In terms of FBPase concentration. [c] The distances were calculated by using eq 2 and a correlation time of 1.8×10^{-9} s. The error limits were calculated by using a 25% deviation in the average value for $1/fT_{1p}$ and the correlation time. Since fT_{1p}/fT_{2p} for the H-8 proton is a factor of ~2, the $1/fT_{1p}$ values may be partially exchange limited, and thus the calculated distance represents a lower limit value.

transitions of enzyme-bound Mn^{2+} (Dwek, 1973). The line width was 30 G. With the assumption that $\tau_c = T_{2e}$ where T_{2e} is the electron-spin relaxation time for Mn^{2+}-FBPase, τ_c was estimated from eq 4

$$1/T_{2e} = [\pi(\text{line width})g(3)^{1/2}]/(7.1 \times 10^{-7})\ \text{G s} \quad (4)$$

where g has the value of 2. From this calculation a lower limit value of 2.2×10^{-9} s was obtained for τ_c. An additional assumption was made that $T_{2e} = T_{1e} = \tau_c$ in order to use eq 2 to calculate Mn^{2+}–nuclei distances (Dwek, 1973). The correlation time was also estimated from the T_{1p}/T_{2p} ratio for the H-8 of AMP at 200 MHz. This ratio was 2.0, and a value of $\tau_c = 0.9 \times 10^{-9}$ s was obtained from eq 5 (Dwek, 1973).

$$\frac{T_{1p}}{T_{2p}} = \frac{4 + 3/(1 + \omega_I{}^2\tau_c{}^2)}{6/(1 + \omega_I{}^2\tau_c{}^2)} \quad (5)$$

The T_{1p}/T_{2p} ratio for the ^{31}P experiments was large (between 100 and 200), indicating that scalar interactions were possibly contributing to $1/T_{2p}$ so that this method for calculating τ_c for Mn^{2+}–^{31}P interactions is unreliable.

The $1/fT_{1p}$ values at 81 MHz for the ^{31}P nuclei of P_i, Fru-6-P, and AMP were not significantly different from the values determined at 40.5 MHz. With the assumption that the correlation time is frequency independent, the τ_c for this interaction is $\leq 1.8 \times 10^{-9}$ s, an upper limit value. The Mn^{2+} to nuclei distances were calculated by using an average of 1.8×10^{-9} s, giving more weight to the values obtained by EPR and ^{31}P NMR. These distances are listed in Tables I–III.

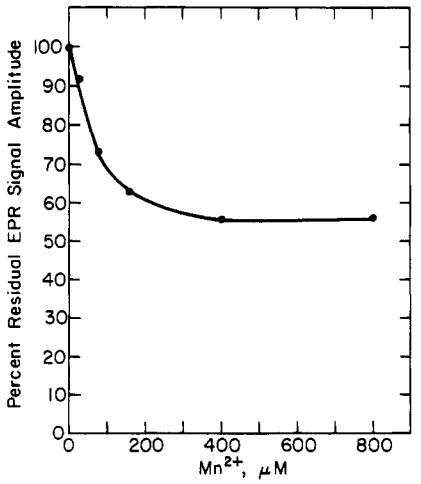

FIGURE 1: Titration of spin-labeled FBPase (32 μM tetramer) with Mn^{2+}. The amplitude of the center line of the EPR spectrum of the spin-labeled FBPase is plotted vs. the concentration of added Mn^{2+}.

Mn^{2+}–Nitroxide Distance. The distance from the bound Mn^{2+} and the one sulfhydryl group of FBPase that can be labeled with a nitroxide spin-label was measured by using the theory of Leigh (1970). The Leigh theory predicts a diminution of the spin-label EPR spectrum upon the addition of a second paramagnetic species, in this case Mn^{2+}. The magnitude of the diminution depends on the distance between the two spins and the correlation time for the interaction. Shown in Figure 1 is a plot of the height of the center line of the nitroxide spectrum vs. the amount of added Mn^{2+}. At saturation the center line is diminished to 56% of its original value. By use of the T_{2e} value determined from the Mn^{2+} EPR spectrum (2.2×10^{-9} s), a Mn^{2+}–nitroxide distance of 16 Å was calculated by using eq 3.

Nitroxide–^{31}P Distance Estimations. The effect of spin-labeled FBPase on the spin–lattice relaxation rates of the ^{31}P nuclei of Fru-6-P and P_i was measured in order to determine the distance between the spin-labeled site and these ligands. There was, however, no change (<25%) in the T_1 values of these nuclei when 20 μM spin-labeled FBPase tetramer and 13.5 mM Fru-6-P and P_i were used. By use of a correlation time of 1×10^{-8} s obtained from the nitroxide spectrum, the distance between the nitroxide and the phosphorus atoms of Fru-6-P and P_i is >9.6 Å.

Discussion

The experiments described herein permit the estimation of distances from the structural metal ion binding site to the phosphorus atoms of Fru-6-P, P_i, and AMP on a given subunit of rabbit liver FBPase. The latter $Mn^{2+}{}_s$ to phosphorus measurement with AMP was refined further by obtaining the effect of $Mn^{2+}{}_s$ on the H-8, H-2, and H-1′ relaxation rates, followed by calculation of their respective distances. In addition, a second locus for mapping allosteric and active site dimensions was sought by spin-labeling the single reactive sulfhydryl group per subunit.

The metal ion to phosphorus distances with P_i and Fru-6-P were found to be 6.3 ± 0.5 Å whereas that to AMP is slightly shorter at 5.2 ± 0.4 Å. These distances are not consistent with direct coordination of $Mn^{2+}{}_s$ to P_i or the phosphoryl groups. Typically these distances lie in the range of 2.8–3.2 Å in inner coordination complexes. The current data should also be compared to the shorter distance of 3.7 ± 0.3 Å measured previously for the $Mn^{2+}{}_s$–Fru-1-P–FBPase complex, this sugar monophosphate being a poor substrate for FBPase (Benkovic et al., 1973). Two interpretations of this difference are that

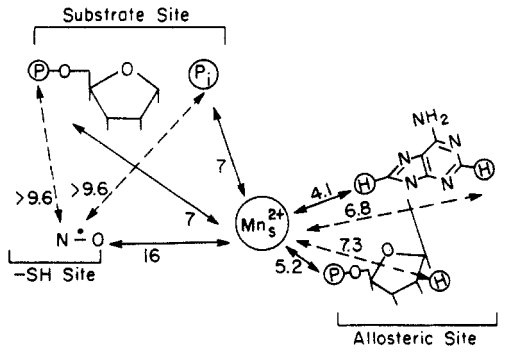

FIGURE 2: Spatial relationships among the active, allosteric, metal ion, and sulfhydryl sites of FBPase. Distances are in angstroms and are not necessarily drawn to scale.

the hydrolytic cleavage of Fru-1,6-P_2 to give P_i increases the Mn^{2+}_s to phosphorus dimension by ~3.0 Å, a lengthening generally encountered for the intervention of a water molecule in a directly coordinated Mn^{2+}_s–phosphate complex, or simply that Fru-1-P binding differs from that Fru-6-P + P_i. Since 4-fold changes in the concentration of added Mn^{2+} did not affect the calculated values of r in the experiments containing Fru-6-P and P_i in 1:1 ratio with FBPase subunits, we conclude that further population of subunits with Mn^{2+}_s (3–4) yields ternary complexes that are similar in their spectrochemical properties and dimensions.

Inclusion of the distances between Mn^{2+}_s and protons on the adenosine moiety allows construction of the skeletal active site–allosteric site model shown in Figure 2. It is of considerable interest that the Mn^{2+}_s ion is approximately equidistant from the two phosphoryl moieties of the product ligands, a feature presumably retained in the substrate complex. The close proximity of AMP suggests that Mn^{2+}_s furnishes, albeit indirectly, a binding site for the modifier as well. The adenosine moiety of the latter is oriented anti on the basis of the decrease in Mn^{2+}_s–^{1}H distance proceeding from H-8 to H-2. One can crudely estimate the dimension of the FBPase subunit as 22 Å by assuming it to be spherical and using a partial specific volume of 0.73 mL/g and subunit molecular weight of 35750. The reactive sulfhydryl is located within this volume 16 Å from the Mn^{2+}_s and apparently does not interact with any of the phosphorus nuclei due to the absence of a measurable effect by the nitroxide spin-label on the ^{31}P relaxation rates. However, orientational constraints acting on the spin-label may be responsible for the absence of an interaction.

An alternative model for the active site–allosteric site–metal ion site depicted in Figure 2 is one in which one of the sites is on a separate subunit. At present, we cannot unambiguously rule out this possibility, but the data in Table II show that progressive occupation of the Mn^{2+}_s site of each of the four subunits results in the same Mn^{2+}_s–nuclei distances. This result suggests the proximity of all three sites on the same subunit.

From the T_{1p} data one can also calculate the minimum dissociation rate constant, $1/\tau_{min}$, for Fru-6-P and P_i. These values are $\geq 4.7 \times 10^5$ s^{-1} for P_i and $\geq 4.0 \times 10^5$ s^{-1} for Fru-6-P (Table I). Since k_{cat} for FBPase hydrolysis in the presence of Mn^{2+} is 10 s^{-1}, the loss of products should not be limiting in the overall reaction cycle.

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