Kinetics and Mechanisms of Activation and Inhibition of Porcine Liver Fructose-1,6-bisphosphatase by Monovalent Cations†

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Received September 13, 1995; Revised Manuscript Received December 14, 1995⊗

ABSTRACT: K^+ and Li^+ were used to study the kinetic effects of monovalent cations on porcine liver fructose-1,6-bisphosphatase (FBPase). At saturating fructose 1,6-bisphosphate (FBP) concentrations, Li^+ was found to be a linear noncompetitive inhibitor with respect to Mg^{2+}. K^+ was found to activate the wild-type enzyme at low concentrations (K_m = 17 mM) and to inhibit the enzyme at high concentrations (K_{IK+} = 68 mM). A steady-state random ter mechanism was proposed, and a mathematical equation was derived to account for the Mg^{2+} and K^+ kinetics and activation of FBPase. Interestingly, when Glu280 was mutated to glutamine by site-directed mutagenesis, K^+ lost the ability to activate the enzyme and became a noncompetitive inhibitor with respect to Mg^{2+}. These kinetic data suggest that K^+ has two distinct sites. One is a high-affinity activation site and the other a low-affinity inhibition site. Glu280 is essential for allowing K^+ to bind at the activation site. Due to the geometric constraints and its small atomic radius, Li^+ can bind only at the inhibitory site. It is postulated that monovalent cations activate FBPase by helping the Arg276 residue "deshield" the partial negative charge on the 1-phosphoryl group of the substrate so that nucleophilic attack on the 1-phosphorus atom is facilitated. In addition, monovalent cations may, along with Mg^{2+} ions and surrounding residues of the protein, help orient the 1-phosphoryl group so as to achieve the optimal position required for catalysis. Monovalent cations inhibit FBPase either by distorting the geometry of the active site or by retarding turnover or product release.

Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) plays an important role in the regulation of gluconeogenesis (Krebs, 1963; Marcus, 1981; Hers & Hue, 1983; Pilkis *et al.*, 1988). In the presence of a divalent metal ion, it catalyzes the hydrolysis of fructose 1,6-bisphosphate (FBP) to form fructose 6-phosphate (F6P) and inorganic phosphate. The reaction is competitively inhibited by fructose 2,6-bisphosphate (Fru-2,6-P_2) and noncompetitively inhibited by AMP (Liu & Fromm, 1990). These two molecules act synergistically to inhibit FBPase (Hers & Van Schaftingen, 1982). AMP is thought to prevent divalent metal binding to FBPase, and divalent metal binding is required for FBPase activity (Gomori, 1943; Benkovic & deMaine, 1982). Fru-2,6-P_2 is believed to have two functions in regulation of FBPase. One is that it is a potent competitive inhibitor of FBP and competes with the substrate for the enzyme's active site (Ke *et al.*, 1989, 1990; Van Schaftingen & Hers, 1980) the other is to keep AMP on the enzyme (Liu & Fromm, 1988), thus enhancing the action of AMP.

It is known that FBPase is a homotetramer with a subunit molecular mass of 37 kDa (Marcus *et al.*, 1982; Burton *et al.*, 1993). It has been shown from binding studies (Benkovic *et al.*, 1978) and the crystallographic structure (Zhang *et al.*, 1993a,b) that there are two divalent metal binding sites per subunit, a high-affinity "structural" site (M1) and a low-affinity "catalytic" site (M2) (Benkovic *et al.*, 1978), and that catalysis requires the formation of a catalytically competent quaternary complex of enzyme−M1−M2−FBP; however, in the case of Mg^{2+} ions, only one ion binds per subunit on the basis of kinetic (Liu & Fromm, 1990) and X-ray diffraction studies (Zhang *et al.*, 1993a,b), and there is no evidence that the M2 site is occupied by Mg^{2+} (Zhang *et al.*, 1993a,b).

A monovalent cation is needed for the enzyme to achieve its maximal activity (Hubert *et al.*, 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus & Hosey, 1980). Among the monovalent cations studied, K^+, NH_4^+, and Tl^+ are the best activators, whereas Li^+ is a strong inhibitor; however, how these monovalent cations affect the activity of FBPase remains unclear. Tejwani *et al.* (1976) proposed that the activation effect of K^+ was to overcome inhibition of the enzyme by high concentrations of divalent metal ions. Xu *et al.* (1993) suggested that activation by monovalent cations might result from the binding of K^+ at a site distinct from the catalytic site. Marcus (1975) reported that activation of FBPase by K^+ could be abolished by modification of arginyl residues with 2,3-butanedione in the presence of AMP. He also found that only one arginyl residue per subunit played an essential role in monovalent cation activation of the enzyme. This arginyl residue was thought to be in the substrate binding site on the basis of the fact that no loss of monovalent cation activation occurred when modification was carried out in the presence of AMP plus the substrate. Very probably, this residue is Arg276, which forms a salt bridge to the 1-phosphoryl group of FBP

† This research was supported in part by Research Grants NS 10546 and GM06920 from the National Institutes of Health, United States Public Health Service, and Grant MCB-9218763 from the National Science Foundation. This is Journal Paper J-16406 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Project 3191.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

0006-2960/96/0435-3038$12.00/0

in the absence of divalent cations (Zhang *et al.*, 1993a,b). This arginyl residue plays important roles in both enzyme activity and Mg^{2+} cooperativity and in determination of the kinetic mechanism of FBPase (Zhang & Fromm, 1995).

In order to gain insight into the effects and mechanism of monovalent cations on FBPase and the location of their binding sites, we have studied the kinetic effects of monovalent cations on FBPase by using K^+ and Li^+ as models of activation and inhibition, respectively. Here, we report that Li^+ is a noncompetitive inhibitor with respect to Mg^{2+}, whereas K^+ activates wild-type FBPase at low concentrations but inhibits the enzyme at high levels. Our results, together with crystallographic studies of FBPase in the presence of monovalent cations (Villeret *et al.*, 1995), identify the different binding sites of K^+ and Li^+. From these studies, the molecular mechanisms of action for these monovalent cations are discussed. This is thought to be the first attempt to explain the role of monovalent cations on FBPase at the molecular level based on the structure and function of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. NADP, FBP, Fru-2,6-P_2, AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. A mutant of recombinant porcine liver FBPase, Glu280Gln, was obtained by site-directed mutagenesis as described elsewhere (Chen *et al.*, 1993). Recombinant and mutant forms of porcine liver FBPase were prepared and purified as previously described (Chen *et al.*, 1993) with slight modifications. The pET-11a expression vector carrying either the wild-type or mutant the FBPase gene was transformed into an *Escherichia coli* DE657 host cell, a strain deficient in the FBPase gene. Mutant forms of the enzyme were obtained in yields comparable to those of the wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (Burton *et al.*, 1993).

Protein Assay. Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism (CD) Spectrometry. CD studies on the wild-type and mutant forms of FBPase were carried out in 50 mM Tris-HCl buffer (pH 7.5) at room temperature in an AVIV CD spectrometer model 62DS kindly supplied by Dr. Earl Stellwagen at the University of Iowa. Samples were placed in a 1 mm cuvette, and data points were collected from 200 to 260 nm in 0.5 nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using a program in the computer of the spectrometer.

Kinetic Studies. FBPase activity during purification and the specific activity of the pure enzyme were measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (Pontremoli & Traniello, 1975). All other kinetic experiments were done using a fluorometric assay (Liu & Fromm, 1990) at pH 7.5 (50 mM Hepes buffer) and 24 °C. The initial rate data were analyzed for kinetic mechanisms by using a MINITAB language program with an α value of 2.0 (Liu & Fromm, 1990).

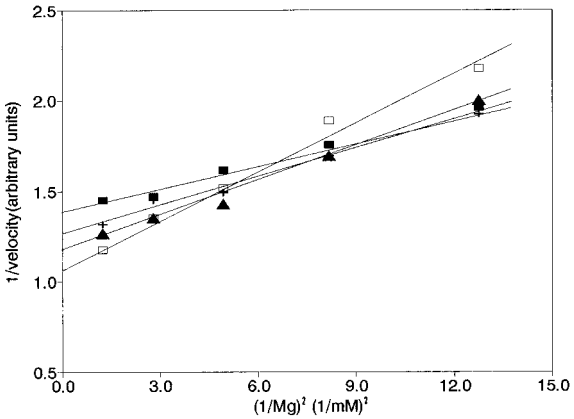


FIGURE 1: Plot of the reciprocal of the initial velocity in arbitrary fluorescent units versus the reciprocal of $[Mg^{2+}]^2$ at different concentrations of K^+ with wild-type FBPase. The concentrations of K^+ are 25 mM (■), 30 mM (+), 35 mM (▲), and 45 mM (□). These concentrations include the amount of K^+ used to adjust the pH of Hepes buffer. The lines are theoretical and are based on eq 3. All the points are experimentally determined.

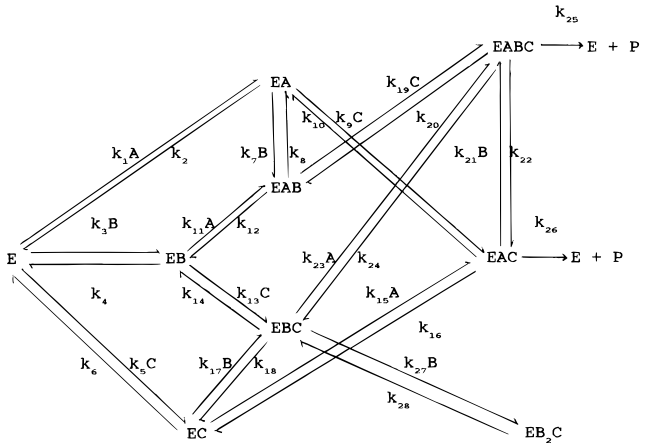
RESULTS

Enzyme Quality. The purities of wild-type recombinant FBPase and the Glu280Gln mutant of porcine FBPase were evaluated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The proteins were greater than 95% pure by using the criterion of electrophoresis. Also, no discernible degradation of the proteins was observed (data not shown).

Secondary Structure Analysis. The secondary structures of recombinant wild-type and the Glu280Gln mutant of FBPase were analyzed by CD spectrometry. The purpose of this study was to determine whether localized or global structural alterations were induced in the mutant. The CD spectral data showed that the spectrum of the mutant was essentially superimposable on that of the wild-type enzyme (data not shown). These results suggest that no major conformational changes occurred in FBPase when Glu280 was mutated to glutamine by using CD as a criterion of secondary protein structure.

Effects of K^+ on Wild-Type FBPase. K^+ plays a dual role in affecting the activity of wild-type FBPase. It can activate and inhibit the enzyme in a concentration-dependent manner. Figure 1 illustrates the effects of K^+ on the kinetics of Mg^{2+} activation and inhibition of FBPase at saturating levels of FBP. From Figure 1, it can be seen that, when $1/v$ vs $1/[Mg^{2+}]^2$ is plotted at different fixed concentrations of K^+, the lines are linear and the intercepts of the lines on $1/v$ axis decrease while the slopes increase as the concentration of K^+ increases. A stochastic approach was used to fit the data shown in Figure 1 and then to explain the results shown in Figure 1. The simplest and most feasible rationale for the data in Figure 1 is described by the mechanism depicted in Scheme 1, which is a steady-state random ter kinetic model. where A = Mg^{2+}, B = K^+, and C = FBP. Scheme 1 depicts a paradigm of K^+ and Mg^{2+} activation of FBPase based upon a number of well-documented investigations reported in the literature. These include the observations that there is an absolute requirement for divalent metal ions by FBPase for activity (Gomori, 1943; Benkovic & deMaine, 1982), that

Scheme 1



monovalent cations such as K^+ can only serve to activate FBPase in the presence of divalent cations (i.e., in the presence of K^+; but in the absence of divalent ions such as Mg^{2+}, FBPase is inactive), that only one Mg^{2+} ion binds per FBPase subunit (Zhang *et al.*, 1993a,b), that the kinetic mechanism of FBPase at pH 9.5 is rapid equilibrium random bi bi (Liu & Fromm, 1990), and that, at saturating Mg^{2+} concentrations, the FBPase reaction in the nonphysiological direction is steady-state random bi bi (Stone & Fromm, 1980). The model described in Scheme 1 was thus proposed in the context of the previously reported kinetic studies on the kinetic mechanism and activation of FBPase by Mg^{2+} and K^+ ions. The initial rate equation for this kinetic model is

$$v = \frac{[k_{25}(\mathrm{EABC}) + k_{26}(\mathrm{EAC})]E_0}{\mathrm{E} + \mathrm{EA} + \mathrm{EB} + \mathrm{EC} + \mathrm{EAB} + \mathrm{EAC} + \mathrm{EBC} + \mathrm{EB_2C} + \mathrm{EABC}} \quad (1)$$

The determinants for E, EA, EB, EC, EAB, EAC, EBC, EB_2C, and EABC were obtained by using a computer program (Fromm, 1975). When these determinants were substituted into eq 1, a very complex equation containing 992 terms in the numerator and 6656 terms in the denominator of eq 1 was obtained (not shown). Since the kinetic studies were performed at a saturating FBP concentration (12 μM), most of the terms in the numerator and denominator were eliminated. To simplify the equation further, four assumptions were made. (a) All forward direction steps are much faster than the reverse direction steps. (b) Mg^{2+} adds more rapidly to the free enzyme and the enzyme−substrate complexes than does K^+. (c) K^+ binds to the enzyme−Mg^{2+}−FBP complex much faster than either the breakdown of the competent quaternary complex of enzyme−Mg^{2+}−FBP−K^+ to products or the dissociation of Mg^{2+} from this complex. (d) K^+ adds to the ternary complex of enzyme, K^+, and FBP to form the dead-end complex EB_2C. These assumptions are based on the kinetics of the system, data from the literature (Gomori, 1943; Benkovic & deMaine, 1982; Hubert *et al.*, 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus & Hosey, 1980), and the crystal structure of FBPase (Zhang *et al.*, 1993a,b) which shows that, in the absence of divalent metal ions, the ϵ-amino group of Arg276 forms a salt bridge with the 1-phosphoryl group of FBP. However, this salt bridge no longer exists in the presence of divalent ions because the bound divalent ions displace the 1-phosphoryl group toward the metal binding site. Thus, formation of a salt bridge between Arg276 and FBP may hinder K^+ binding to the free enzyme, but the binding of Mg^{2+} to the enzyme disrupts this salt bridge, which provides room for K^+, so that K^+ binding is facilitated. On the basis of these assumptions, the complex equation was simplified to eq 2:

$$\frac{k_{25}E_0}{v} = 1 + \frac{k_{22}/k_{21}}{k_{26}/k_{21} + \mathrm{B}} + \frac{k_{17}/k_{25}}{k_{15}k_{23}}\left(\frac{k_{18}/k_{17} + \mathrm{B}}{A^2}\right)\left(1 + \frac{B}{K_{\mathrm{IB}}}\right) \quad (2)$$

in which $k_{21} \gg k_{26}$ and $k_{17} \gg k_{18}$ on the basis of the above assumptions. Thus, eq 2 is further simplified to eq 3:

$$\frac{1}{V} = \frac{1}{V_{\mathrm{m}}}\left[1 + \frac{K_{\mathrm{a}}B}{A^2}\left(1 + \frac{B}{K_{\mathrm{IB}}}\right) + \frac{K_{\mathrm{b}}}{B}\right] \quad (3)$$

where v, A, and B represent the initial velocity, the concentration of Mg^{2+}, and the concentration of K^+, respectively: $V_{\mathrm{m}} = k_{25}E_0$, which is the maximum velocity; $K_{\mathrm{a}} = k_{17}k_{25}/k_{15}k_{23}$; K_{b} is the activation constant for K^+; and K_{IB} is the inhibition constant for K^+. The data in Figure 1 fit very well to eq 3; e.g., the goodness of fit was 1.5%. The kinetic constants for the effects of K^+ on wild-type FBPase are summarized in Table 1. From Table 1, it can be seen that K_{IB} is about 4-fold higher than K_{b}.

Table 1: Kinetic Constants for the Effects of K^+ on Wild-Type FBPase

constant	enzyme−ligand interaction	value (mM)
K_{b}	FBP−E−Mg^{2+} + K^+ = K^+−E−Mg^{2+}−FBP	17 ± 0.23
K_{IB}	K^+−E−FBP + K^+ = K_2^+−E−FBP	68 ± 2.1

The data in Figure 1 are not consistent with a rapid equilibrium random model in which all the steps in Scheme 1 are in rapid equilibrium relative to the breakdown of the EAC and EABC complexes to products. In addition, the data do not fit to the model where B binds the EABC complex, i.e., K^+ and Mg^{2+} bind mutually exclusively at site 1.

Effects of K^+ on Glu280Gln Mutant FBPase. Crystallographic studies have shown (Figure 4) that metal site 1 is defined by Glu280, Glu97, Asp118, Asp121, and the 1-phosphoryl group of FBP (Zhang *et al.*, 1993a,b); metal site 3, which is thought to be the putative K^+ activation site, is defined by Glu280, Arg276, and the 1-phosphoryl group of FBP (Villeret *et al.*, 1995). Therefore, Glu280 is thought to be important for metal ion action. A mutant, Glu280Gln, was prepared and characterized, and the kinetic effects of K^+ on this mutant form of FBPase were studied. The mutation caused a dramatic decrease in enzyme activity, i.e., the k_{cat} of the mutant enzyme is 0.015 s^{-1}, which is 0.1% of that of the wild-type enzyme. The kinetic data are shown in Figure 2. From Figure 2, it can be seen that, when $1/v$ vs $1/[Mg^{2+}]$ is plotted at different fixed concentrations of K^+, a noncompetitive inhibition pattern was obtained. These findings suggest that K^+ is a noncompetitive inhibitor with respect to Mg^{2+} when Glu280 of FBPase is changed to glutamine. The data in Figure 2 fit well to eq 4 (the goodness of fit was 5.3%) but did not fit to other models such as a competitive inhibition model, a rapid equilibrium random bi bi model, or the steady-state random model shown in

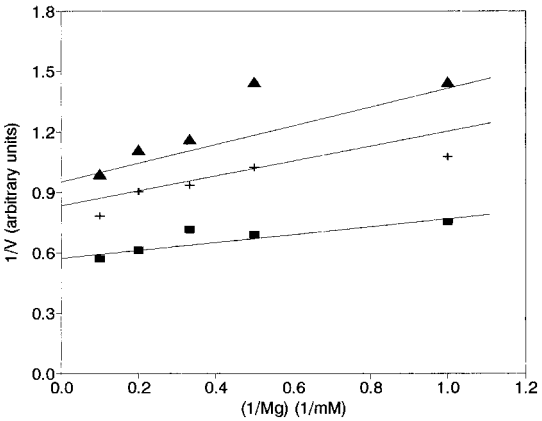


FIGURE 2: Plot of the reciprocal of the initial velocity in arbitrary fluorescent units vs the reciprocal of $[Mg^{2+}]$ at different concentrations of K^+ with the Glu280Gln mutant FBPase. The concentrations of K^+ are 25 mM (■), 125 mM (+), and 175 mM (▲). These concentrations include the amount of K^+ used to adjust the pH of Hepes buffer. The lines are theoretical and are based on eq 4, and the points are experimentally determined.

Table 2: Kinetic Constants for the Effects of K^+ on Glu280Gln FBPase

constant	enzyme−ligand interaction	value (mM)
K_a	$E + Mg^{2+} = E{-}Mg^{2+}$	1.4 ± 0.14
K_i	$E + K^+ = K^+{-}E$	102 ± 56.0
K_{ii}	$E{-}Mg^{2+} + K^+ = K^+{-}E{-}Mg^{2+}$	222 ± 43.0
K_{iii}	$K^+{-}E + Mg^{2+} = K^+{-}E{-}Mg^{2+}$	3.4 ± 0.51

Scheme 1. The form of eq 4 is

$$\frac{1}{v} = \frac{1}{V_m}\left(1 + \frac{I}{K_{ii}} + \frac{K_a}{A} + \frac{K_a I}{K_i A}\right) \quad (4)$$

where v, V_m, A, I, K_i, and K_{ii} represent the initial velocity, the maximum velocity, the concentration of Mg^{2+}, the concentration of K^+, and the inhibition constants for K^+, respectively. It is noteworthy that the A term in eq 4 is first power instead of second power. These results suggest that the mutation caused FBPase to lose Mg^{2+} sigmoidicity. When the concentration of Hepes-Na^+ buffer (pH 7.5) was varied from 30 to 250 mM, enzyme activity was not affected. This result indicates that the effect of K^+ on Glu280Gln FBPase is not due to ion strength.

Equation 4 is the rate equation derived from a conventional noncompetitive inhibition model. The kinetic constants for the effect of K^+ on Glu280Gln mutant FBPase are shown in Table 2.

Effects of Li^+ on Wild-Type FBPase. Figure 3 depicts the kinetic effects of Li^+ on wild-type FBPase. At a saturating FBP concentration (12 μM), a noncompetitive inhibition pattern was obtained when $1/v$ was plotted vs $1/[Mg^{2+}]^2$. The data in Figure 3 gave excellent fits to eq 4 where the A term was raised to the second power (Mg^{2+} sigmoidicity was not affected by Li^+ ions). The goodness of fit was 3.0%. When the data in Figure 3 were fit to other models such as a competitive inhibition model, a rapid equilibrium random bi bi model, or the steady-state random model as shown in Scheme 1, the goodness of fit value was at least 20%. The kinetic constants for the effects of Li^+ on FBPase are summarized in Table 3. From Tables 1 and 3, it can be seen that the affinity of FBPase is much higher for Li^+ than for K^+. Because of its noncompetitive nature, Li^+ and Mg^{2+} are not mutually exclusive binding ligands, and a complex of the two cations in the region of site 1 is consistent with both the kinetic data of this report and the structural findings of Villeret *et al.* (1995).

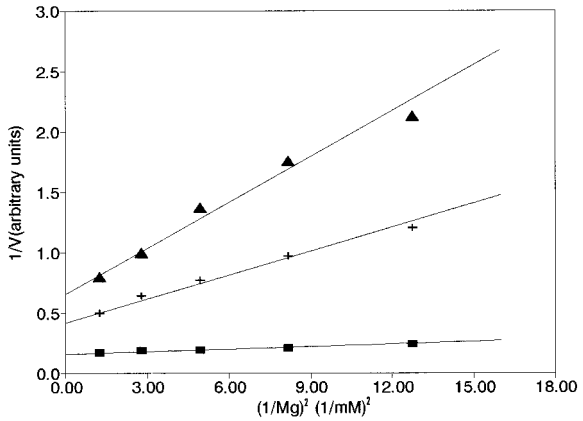


FIGURE 3: Plot of the reciprocal of the initial velocity in arbitrary fluorescent units vs the reciprocal of $[Mg^{2+}]^2$ at different concentrations of Li^+ with wild-type FBPase. The concentrations of Li^+ are 0 mM (■), 2 mM (+), and 4 mM (▲). The lines are theoretical and are based on eq 4, where the A terms are second power, and the points are experimentally determined. The assay solutions contained 25 mM K^+ which was used to adjust the pH of Hepes buffer. When 50 mM Tris-HCl buffer (pH 7.5) was used (no K^+), identical results were obtained.

Table 3: Kinetic Constants of the Effects of Li^+ on Wild-Type FBPase

constant	enzyme−ligand interaction	value
K_a	$E + Mg^{2+} = E{-}Mg^{2+}$	0.50 ± 0.04 mM^2
K_i	$E + Li^+ = Li^+{-}E$	0.20 ± 0.05 mM
K_{ii}	$E{-}Mg^{2+} + Li^+ = Li^+{-}E{-}Mg^{2+}$	1.3 ± 0.57 mM
K_{iii}	$Li^+{-}E + Mg^{2+} = Li^+{-}E{-}Mg^{2+}$	3.3 ± 0.05 mM

DISCUSSION

X-ray diffraction data have established that there are three metal binding sites (Figure 4) associated with each FBPase subunit, all of which can bind monovalent cations (Villeret *et al.*, 1995). Two of these sites, designated as sites 1 and 2, are divalent metal binding sites when the metals are Mn^{2+} or Zn^{2+} (Zhang *et al.*, 1993a,b), whereas site 3 reported for FBPase is specific for monovalent cations. There is no evidence that Mg^{2+} binds to site 2 (Zhang *et al.*, 1993a,b; Benkovic & deMaine, 1982). In the presence of Mg^{2+} and activating concentrations of K^+, sites 2 and 3 are expected to be occupied by K^+. It has been shown that K^+ activation of FBPase is similar with either Mg^{2+} or Mn^{2+} (Hubert *et al.*, 1970). Tight binding at site 2 by Mn^{2+} would preclude inhibition at this site by K^+. Because Li^+ binds exclusively at site 1 (Villeret *et al.*, 1995) to inhibit FBPase, it is not unreasonable to assume that site 1 may be the low-affinity K^+ inhibition site.

The results of this report are consistent with the chemical modification studies reported by Marcus (1975) and the crystallographic studies from Lipscomb's laboratory (Villeret *et al.*, 1995). Marcus found that modification of pig kidney FBPase with 2,3-butanedione in the presence of AMP resulted in the loss of activation of the enzyme by monovalent cations, but no loss of monovalent cation activation was observed when the modification was carried out in the presence of both AMP and FBP. In addition, Marcus found

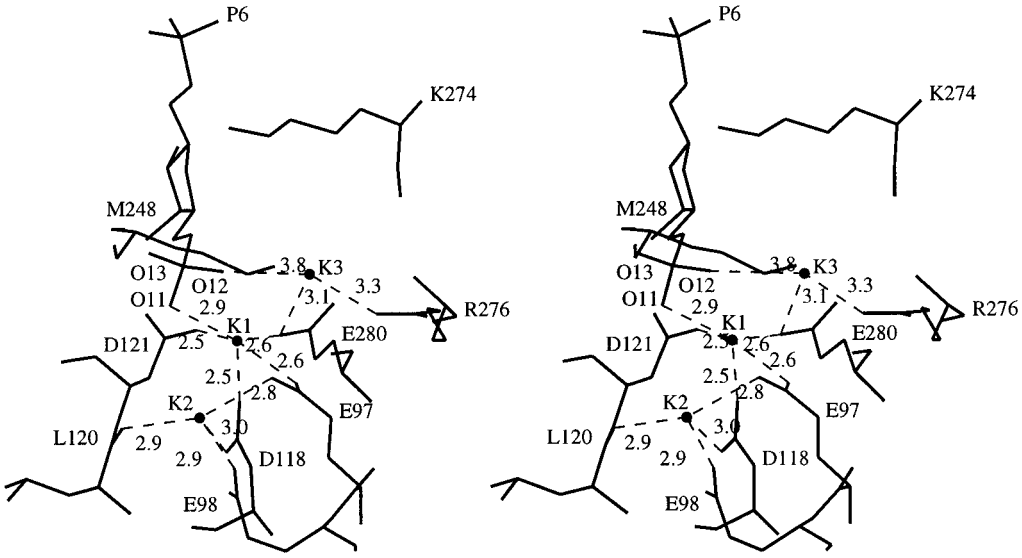


FIGURE 4: Stereoview of the metal binding region in the presence of the substrate analogue anhydro-D-glucitol-1,6-bisphosphate (AhG-1,6-P_2) and potassium ions, as observed in the crystal structure (Villeret *et al.*, 1995). P6 refers to the 6-phosphoryl group of AhG-1,6-P_2; O11, O12, and O13 are 1-phosphoryl oxygens. K1, K2, and K3 refer to potassium ions at sites 1, 2, and 3, respectively. Distances between K^+ ions and their ligands are indicated. K1 and K2 are bound at the divalent metal sites, and K3 is bound at the specific site for K^+ or Tl^+ ions. K1 and K2 are 3.9 Å apart; K1 and K3 are 3.4 Å apart.

that one arginyl residue per subunit plays an essential role in monovalent cation activation of the enzyme. It is now clear that this residue is probably Arg276 because this arginyl residue alone, together with Glu280 and the 1-phosphoryl group of FBP, defines the K^+ and Tl^+ binding site (Villeret *et al.*, 1995).

Li^+ cannot be seen by X-ray analysis, but kinetic evidence in this report and indirect evidence from the crystal structure data (Villeret *et al.*, 1995) have shown that it binds at metal site 1, which is defined by Glu280, Glu97, Asp118, Asp121, and the 1-phosphoryl group of FBP (Zhang *et al.*, 1993a,b; Villeret *et al.*, 1995). The kinetic data presented in this report suggest that K^+ can bind at two sites: one activation site with high affinity and one inhibition site with low affinity. It is probable that metal site 3 is the site where monovalent cations bind and activate FBPase (X-ray analysis has shown that metal site 3 is the only site specific for K^+, Tl^+, and presumably NH_4^+ ions), whereas metal site 1 is very probably the site where monovalent cations bind and inhibit the enzyme. In this case, monovalent cations may either replace the divalent cation at this site or coexist with the divalent cation in this negatively charged pocket. Our kinetic data suggest that the former is true for K^+ inhibition, and the latter may be true for Li^+ inhibition. These arguments are supported by the finding that the effects of monovalent cations on FBPase are size-dependent (Nakashima & Tuboi, 1976).

The kinetic data shown in Figure 2 also suggest that, when Glu280 was mutated to glutamine, K^+ lost the ability to activate the enzyme and became a noncompetitive inhibitor with respect to Mg^{2+}. Presumably, K^+ can only bind to metal site 1 of the mutant form of FBPase and inhibit the enzyme. This is deduced from crystal structure data (Villeret *et al.*, 1995) showing that Li^+ binds to site 1 and the kinetic findings in this report showing that Li^+ is a noncompetitive inhibitor with respect to Mg^{2+}. Metal site 1 of the mutant enzyme may not be defined as well as it is in the wild-type enzyme so that K^+ may coexist with Mg^{2+} at this site and thus play a role as a noncompetitive inhibitor. Li^+ is the only monovalent cation known to have only inhibitory effects on FBPase. From Figure 3, it can be seen that, at saturating levels of FBP, Li^+ is a noncompetitive inhibitor with respect to Mg^{2+}. Possibly due to the geometric constraints at the active site of the enzyme and the ionic radius of the cation, Li^+ may coexist with Mg^{2+} at site 1 and may inhibit the enzyme by disturbing the geometry of Mg^{2+} and its ligands in a manner similar to that in which K^+ pushes the guanidinium group of Arg 276 aside at site 3 (Villeret *et al.*, 1995).

The effects of monovalent cations on FBPase have long been recognized (Hubert *et al.*, 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus & Hosey, 1980; Nakashima & Tuboi, 1976), but the mechanism of action of these ions remains unknown. It is well-known that monovalent cations have similar effects on Hsc70 protein (O'Brien & Mckay, 1995) and on FBPase. The difference between Hsc70 and FBPase is that there is no positive residue at the K^+ binding site in Hsc70, whereas FBPase has Arg276 at this site. In the case of Hsc70 protein, K^+ participates directly in the hydrolysis of ATP. It facilitates the hydrolysis either by deshielding the target phosphorus atom and making it more susceptible to nucleophilic attack or by electrostatically stabilizing the pentavalent transition state (Wilbanks & Mckay, 1995). Some proteins have positive residues at their active site which play the role of monovalent cations. For example, the ϵ-amino group of Lys18 in actin forms a salt bridge to oxygen of ADP (Kabsch *et al.*, 1990), and Lys16 within the P-loop of H-ras p21 (Pai *et al.*, 1990) has been shown to fulfill the role of K^+. However, in the case of FBPase, Arg276 and K^+ cannot replace each other, and both must be present for the enzyme to reach its optimal activity. When Arg276 was mutated to methionine, the enzyme lost >99% of its activity (Zhang & Fromm, 1995), yet K^+ can still activate but cannot completely restore the activity of the mutant FBPase (R. Zhang and H. J. Fromm, unpublished observations). This suggests that Arg276 plays an important role in enzyme activity. This residue is also known to be essential for Mg^{2+} sigmoidicity, and it deter-

mines the kinetic mechanism of FBPase (Zhang & Fromm, 1995).

On the basis of our kinetic data and the X-ray structure data obtained by Villeret *et al.* (1995), it is postulated that metal site 3 of FBPase is the monovalent cation activation site where monovalent cations such as K^+, Tl^+, or NH_4^+, together with Arg276, activate the enzyme by deshielding the 1-phosphoryl group of the substrate and aid in the nucleophilic attack at the phosphorus atom by OH^-. In addition, this region of FBPase is involved with Mg^{2+} ions and surrounding residues of the protein in maintaining a protein conformation that allows the 1-phosphoryl group of FBP to achieve the optimal position required for catalysis. On the other hand, metal site 1 of the enzyme is the site responsible for monovalent cation inhibition of the enzyme. Monovalent cations inhibit FBPase at site 1 either by distorting the geometry of the active site or by retarding product release in a manner similar to the inhibitory effect of Li^+ on inositolmonophosphatase (Pollack *et al.*, 1994) which is similar in structure to FBPase (Zhang *et al.*, 1993a,b). From the perspective of kinetics, FBPase complexed with Li^+ loses its ability to carry out catalysis, i.e., it forms a dead-end complex. The inhibitory effects of Li^+ on inositolmonophosphatase may be the basis for the clinical treatment of manic depression. The effects of monovalent cation inhibition, and/or activation of FBPase, may represent a common phenomenon associated with a number of phosphotransferase enzymes.

REFERENCES

Benkovic, P. A., Caperelli, C. A., deMaine, M. M., & Benkovic, S. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2185−2189.

Benkovic, S. J., & deMaine, M. M. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 45−82.

Burton, V. A., Chen, M., Ong, W. C., Ling, T., Fromm, H. J., & Stayton, M. M. (1993) *Biochem. Biophys. Res. Commun. 192*, 511−517.

Chen, L., Remesh, H., Chen, M., & Fromm, H. J. (1993) *Arch. Biochem. Biophys. 307*, 350−354.

Colombo, G., & Marcus, F. (1973) *J. Biol. Chem. 248*, 2743−2745.

Fromm, H. J. (1975) *Initial Rate Enzyme Kinetics*, pp 295−306, Springer-Verlag, Berlin.

Gomori, G. (1943) *J. Biol. Chem. 148*, 139−149.

Hers, H. G., & Van Schaftingen, E. (1982) *Biochem. J. 206*, 1−12.

Hers, H. G., & Hue, L. (1983) *Annu. Rev. Biochem. 52*, 617−653.

Hubert, E., Villanueva, J., Gonzalez, A. M., & Marcus, F. (1970) *Arch. Biochem. Biophys. 138*, 590−597.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37−44.

Ke, H., Thorpe, C. M., Seaton, B. A., Marcus, F., & Lipscomb, W. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1475−1479.

Ke, H., Zhang, Y., & Lipscomb, W. N. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5243−5247.

Krebs, H. A. (1963) in *Advances in Enzyme Regulation* (Weber, G., Ed.) Vol. 1, pp 385−400, Pergamon Press Ltd., London.

Liu, F., & Fromm, H. J. (1988) *J. Biol. Chem. 263*, 9122−9128.

Liu, F., & Fromm, H. J. (1990) *J. Biol. Chem. 265*, 7401−7406.

Marcus, F. (1975) *Biochemistry 14*, 3916−3921.

Marcus, F. (1981) in *The Regulation of Carbohydrate Formation and Utilization in Mammals* (Veneziabe, C. M., Ed.) pp 269−290, University Park Press, Baltimore.

Marcus, F., & Hosey, M. M. (1980) *J. Biol. Chem. 255*, 2481−2486.

Marcus, F., Edelstein, L., Reardon, I., & Heinrikson, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7161−7165.

Nakashima, K., & Tuboi, S. (1976) *J. Biol. Chem. 251*, 4315−4321.

O'Brien, M. C., & Mckay, D. B. (1995) *J. Biol. Chem. 270*, 2247−2250.

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351−2359.

Pilkis, S. J., El-Maghrabi, M. R., & Claus, T. H. (1988) *Annu. Rev. Biochem. 57*, 755−783.

Pollack, S. J., Atack, J. R., Knowles, M. R., McAllister, G., Ian Ragen, C., Baker, R., Fletcker, S. R., Iversen, L. L., & Broughton, H. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5766−5770.

Pontremoli, S., & Traniello, S. (1975) *Methods Enzymol. 42*, 369−374.

Stone, S. R., & Fromm, H. J. (1980) *Biochemistry, 19*, 620−625.

Tejwani, G. A., Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1976) *Arch. Biochem. Biophys. 177*, 255−264.

Van Schaffingen, E., & Hers, H. G. (1980) *Biochem. Biophys. Res. Commun. 96*, 1524−1531.

Villanueva, J., & Marcus, F. (1974) *J. Biol. Chem. 249*, 745−749.

Villeret, V., Huang, S., Fromm, H. J., & Lipscomb, W. N. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8916−8920.

Wilbanks, S. M., & Mckay, D. B. (1995) *J. Biol. Chem. 270*, 2251−2257.

Xu, G., Yu, Z., Hu, G., & Cao, H. (1993) *Biochem. Biophys. Res. Commun. 194*, 1483−1490.

Zhang, R., & Fromm, H. J. (1995) *Biochemistry 34*, 8190−8195.

Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., & Lipscomb, W. N. (1993a) *Biochemistry 32*, 1844−1857.

Zhang, Y., Liang, J.-Y., & Lipscomb, W. N. (1993b) *Biochem. Biophys. Res. Commun. 190*, 1080−1083.

BI952188I