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Crystallographic evidence for the action of potassium, thallium, and lithium ions on fructose-1,6-bisphosphatase

(x-ray crystallography/allosteric enzyme/binding sites)

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ABSTRACT **Fructose-1,6-bisphosphatase (Fru-1,6-Pase; D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) requires two divalent metal ions to hydrolyze α-D-fructose 1,6-bisphosphate. Although not required for catalysis, monovalent cations modify the enzyme activity; K^+ and Tl^+ ions are activators, whereas Li^+ ions are inhibitors. Their mechanisms of action are still unknown. We report here crystallographic structures of pig kidney Fru-1,6-Pase complexed with K^+, Tl^+, or both Tl^+ and Li^+. In the T form Fru-1,6-Pase complexed with the substrate analogue 2,5-anhydro-D-glucitol 1,6-bisphosphate (AhG-1,6-P_2) and Tl^+ or K^+ ions, three Tl^+ or K^+ binding sites are found. Site 1 is defined by Glu-97, Asp-118, Asp-121, Glu-280, and a 1-phosphate oxygen of AhG-1,6-P_2; site 2 is defined by Glu-97, Glu-98, Asp-118, and Leu-120. Finally, site 3 is defined by Arg-276, Glu-280, and the 1-phosphate group of AhG-1,6-P_2. The Tl^+ or K^+ ions at sites 1 and 2 are very close to the positions previously identified for the divalent metal ions. Site 3 is specific to K^+ or Tl^+. In the divalent metal ion complexes, site 3 is occupied by the guanidinium group of Arg-276. These observations suggest that Tl^+ or K^+ ions can substitute for Arg-276 in the active site and polarize the 1-phosphate group, thus facilitating nucleophilic attack on the phosphorus center. In the T form complexed with both Tl^+ and Li^+ ions, Li^+ replaces Tl^+ at metal site 1. Inhibition by lithium very likely occurs as it binds to this site, thus retarding turnover or phosphate release. The present study provides a structural basis for a similar mechanism of inhibition for inositol monophosphatase, one of the potential targets of lithium ions in the treatment of manic depression.**

Fructose-1,6-bisphosphatase (Fru-1,6-Pase; D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), a key enzyme in the gluconeogenesis pathway, catalyzes the hydrolysis of α-D-fructose 1,6-bisphosphate (Fru-1,6-P_2) to α-D-fructose 6-phosphate (Fru-6-P) and inorganic phosphate. Divalent metal ions, such as Mg^{2+}, Mn^{2+}, or Zn^{2+}, are a strict requirement for catalytic activity (1–3). The enzyme is regulated by two inhibitors: AMP, which binds at an allosteric site, and fructose 2,6-bisphosphate (Fru-2,6-P_2), which binds to the active site (3–6). These two inhibitors act synergistically to regulate Fru-1,6-Pase activity in gluconeogenesis.

Pig kidney Fru-1,6-Pase is a tetramer with D2 symmetry. Crystallographic structures of the allosteric states of the enzyme established two quaternary conformations, the R and T forms, which differ by a 15–17° rotation of the lower dimer C3C4 relative to the upper dimer C1C2. R form structures currently include Fru-1,6-Pase complexed with Fru-6-P (7), Fru-2,6-P_2 (8), Fru-1,6-P_2, 2,5-anhydro-D-glucitol 1,6-bisphosphate (AhG-1,6-P_2), and 2,5-anhydro-D-mannitol 1,6-bisphosphate in either the presence or absence of divalent metal cations (9). T form structures include the AMP complex (10), the Fru-6-P–AMP–Mg^{2+} complex (11), the Fru-2,6-P_2–AMP–Zn^{2+} complex (12), and the AhG-1,6-P_2–AMP–Mn^{2+} complex (13).

Only divalent metal ions are required for catalysis. However, monovalent cations, such as K^+ or NH_4^+, have some effect on mammalian Fru-1,6-Pase activity and increase the maximal velocity (14–17). Marcus and Hosey (18) showed, in a study of rat liver Fru-1,6-Pase, that thallium is also an activator of Fru-1,6-Pase; V_{max} in the presence of Tl^+ was equal to that obtained with K^+, but the affinity of the enzyme for Tl^+ (K_a = 16 mM) was 3 times greater than for K^+. On the other hand, Li^+ is an inhibitor of Fru-1,6-Pase, and among all the monovalent cations tested so far it is the only one to inhibit the enzyme. Although these effects of monovalent cations on mammalian Fru-1,6-Pases have been known for a long time, their mode of action on the enzymes is still not understood. Interestingly, inositol monophosphatase (IMPase), which shares a similar topology of secondary structures with Fru-1,6-Pase (19), also requires divalent cations (20, 21) and is similarly affected by monovalent cations (22). Inhibition of IMPase by Li^+ is of particular interest, since this enzyme is one of the potential targets of lithium ions used to attenuate the phosphatidylinositol cycle activity in the therapeutic treatment of manic depression (23). Based on modeling, kinetic, and mutagenesis studies, it has been suggested that Li^+ might bind at one of the two divalent cation binding sites after hydrolysis and inositol departure, retarding phosphate release (21). Crystallographic structures in the presence of other monovalent cations such as K^+ or Tl^+ have not been reported.

The present studies were initiated in order to reveal the changes that occur upon binding of monovalent cations to Fru-1,6-Pase. We report here the structures of Fru-1,6-Pase complexed with Tl^+ (R form) or with AMP, AhG-1,6-P_2 and Tl^+, K^+, or both Tl^+ and Li^+ (T form).§ The thallium binding sites were found at similar positions in the R and T forms of Fru-1,6-Pase. Consequently, most of our studies were done on the T form crystals, which diffract to a resolution of 2.2–2.3 Å, compared with only 3.0 Å resolution for our current R form crystals.

Three K^+ or Tl^+ binding sites are identified in the active sites. Two sites (referred to as sites 1 and 2) correspond to the divalent metal positions previously found in the Mn^{2+} complexes. A third metal site (site 3) is present, which is 3.4 Å away from the metal ion at site 1. This site is defined by the 1-phosphate group of the substrate analogue, the carboxylate group of Glu-280 (a residue that is also a ligand of metal site 1), and is near the guanidinium group of Arg-276. In the absence of Tl^+ or K^+, site 3 is occupied by the guanidinium

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Abbreviations: Fru-1,6-Pase, fructose-1,6-bisphosphatase; AhG-1,6-P_2, 2,5-anhydro-D-glucitol 1,6-bisphosphate; IMPase, inositol monophosphatase.

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§The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1FPI, 1FPJ, 1FPK, 1FPL).

group of Arg-276, which, when mutated to Met, causes a striking decrease in activity, a loss of cooperativity for Mg^{2+}, and a change in the catalytic mechanism (24). These observations suggest that Tl^+ or K^+ can play a direct role in catalysis, replacing Arg-276 in the active site.

In the presence of Li^+ ions, Tl^+ at site 1 is no longer observed; Tl^+ at sites 2 and 3 is not affected. As site 1 is the high-affinity divalent metal site, it is suggested that inhibition of Fru-1,6-Pase results from the binding of Li^+ at this site, thus retarding turnover or phosphate release. Possible mechanisms of inhibition by Li^+ are briefly discussed.

MATERIALS AND METHODS

Crystal Structure Determinations. Pig kidney Fru-1,6-Pase was purified according to the procedures of Ke *et al.* (7, 25). AhG-1,6-P_2 was synthesized according to Hartman and Barker (26). R and T form crystals of Fru-1,6-Pase were obtained by dialyzing the enzyme at a concentration of 10–15 mg/ml against a buffer containing 20 mM Tris base, 4% (R form) or 11–12.5% (T form) PEG 3350, 5.0 mM NaN_3, 1.0 mM 2-mercaptoethanol, 0.1 mM maleic acid, 1 mM AhG-1,6-P_2 (T form), and 1 mM AMP (T form).

Crystals of the R form enzyme complexed with Tl^+ ions were prepared by soaking the native crystals in a buffer containing 20 mM Tris base, 2.5 mM maleic acid, 15% PEG 3350, and 10 mM $TlNO_3$ at pH 7.4 for 4 days. Crystals of the T form enzyme complexed with AMP, AhG-1,6-P_2, and thallium ions were obtained by cocrystallizing the enzyme in the presence of 5 mM Tl_2SO_4. Some T form crystals of the enzyme complexed with thallium ions were subsequently soaked for 3 days in the crystallization buffer but in the presence of 5 mM Li_2SO_4. The potassium complex was prepared by soaking crystals of the T form in the buffer described above but in the presence of 100 mM K_2SO_4.

X-ray diffraction data were collected on a Siemens X-1000 multiwire area detector at the Gibbs Laboratory of Harvard University and were reduced by using the program XDS (27). For the R form crystals, the diffraction data extended only to 3 Å resolution. Structural comparison between the R and T form structures complexed with thallium ions showed essentially similar metal binding sites in both structures. Consequently, T form crystals were preferentially used because of their higher resolution (2.2–2.3 Å). The space groups of the R and T form crystals are $P3_221$ and $P2_12_12$, respectively. Two monomers are present in the asymmetric unit of both crystal forms. Table 1 summarizes the different parameters for the R form crystal (Tl^+ complex) and the three T form crystals (Tl^+, K^+, and Tl^+Li^+ complexes) used in this study.

The coordinates of the unligated R form of Fru-1,6-Pase (7) were used as a starting model to determine the R form–Tl^+ complex. For the T form structures, coordinates of the Fru-1,6-Pase–AMP–AhG-1,6-P_2–Mn^{2+} complex (13) were used. Parameters for AhG-1,6-P_2 and AMP were obtained with QUANTA (28). Positional refinements were carried out with X-PLOR (29). Metal ions were located from $F_o - F_c$ Fourier maps contoured at a high level (5σ). A charge of zero was assigned to the metal ions in order to avoid undue bias arising from the long-range Coulomb potential. To avoid any bias, metal–ligand distances were not restrained in the refinements. Those solvent molecules that showed well defined density (at least 3.5σ above the mean value) were included in the T form structures. Water molecules that had B factors greater than 55 $Å^2$ after the first cycle of temperature factor refinement were deleted. Temperature and occupancy factors for the metal ions were refined alternatively in the T form structures until convergence was reached. Temperature factors for the metal ions were kept fixed at a value of 40 $Å^2$ in the R form structure as occupancy factors were subsequently refined. The statistics for data collection, reduction, and refinements are given in Table 1. Due to lack of well defined electron density the 8 N-terminal residues and also the 62–71 loops were not included in the T form structures, and residues 1–6 and 55–71 were not included in the R form structure.

Table 1. Summary of crystallographic parameters for data collection, reduction, and refinements

	Tl^+ (R)	Tl^+ (T)	K^+ (T)	Tl^+Li^+ (T)
Space group	$P3_221$	$P2_12_12$	$P2_12_12$	$P2_12_12$
a, Å	132.6	61.1	61.2	61.2
b, Å	132.6	166.9	167.1	166.8
c, Å	67.7	80.2	80.0	80.3
Total no. of reflections	33,304	94,731	98,031	93,824
No. of unique reflections	13,824	30,726	29,101	37,484
R_{merge}	0.091	0.089	0.072	0.066
Resolution, Å	3.0	2.3	2.3	2.2
Completeness, %	73	88	83	95
rms of bond lengths, Å	0.013	0.011	0.009	0.011
rms of angles, degrees	2.11	1.68	1.49	1.64
R factor	0.226	0.194	0.199	0.189
H_2O, mol/ asymmetric unit	0	127	111	122

Tl^+ (R), R form–Tl^+ complex; Tl^+ (T), K^+ (T), and Tl^+Li^+ (T), T form structures complexed with Tl^+, K^+, or both Tl^+ and Li^+ ions, respectively.

RESULTS

Crystal Structures of Fru-1,6-Pase Complexed with Tl^+, K^+, and Tl^+Li^+. Monovalent cations were identified from difference Fourier maps in the active site of Fru-1,6-Pase. In previous crystallographic studies two divalent metal binding sites were reported in the active site of the enzyme, in either the T or the R state, when the substrate analogue AhG-1,6-P_2 was bound (9, 13). In summary, site 1 is defined by residues Glu-97, Asp-118, Asp-121, Glu-280, and a 1-phosphate oxygen of AhG-1,6-P_2. Site 2 is defined by residues Glu-97, Glu-98, Asp-118, Leu-120, and also a 1-phosphate oxygen of AhG-1,6-P_2. The metal ion at site 1 is similarly coordinated in the T and R forms. However, the metal ion at site 2 in the T form structure is slightly displaced and is no longer coordinated to a 1-phosphate oxygen. These changes in metal binding positions relative to the 1-phosphate group of the substrate explain the allosteric inhibition that occurs upon binding of AMP to Fru-1,6-Pase (13, 30).

Thallium Binding Sites. In the R form–Tl^+ structure (without AhG-1,6-P_2), three thallium ions are present in the active sites. Two of them are bound at the divalent metal sites. The third thallium site, adjacent to site 1, is 2.9 Å away from the carboxylate group of Glu-280. Thallium ions at sites 1 and 3 are 3.6 Å apart.

Three thallium ions are also present in the active site of the T form structure, in the presence of AhG-1,6-P_2 (Fig. 1). Two Tl^+ are bound at the divalent metal sites, although slight differences are observed in the coordination and position of the Tl^+ ions when compared to those of the Mn^{2+} ions previously identified (13). Tl^+ at site 1 is coordinated to Glu-97, Asp-118, Asp-121, and Glu-280. The average distance between the carboxylate group of these residues and this thallium ion is 2.5 ± 0.1 Å. However, the distance between the closest 1-phosphate oxygen and this Tl^+ is 2.9 ± 0.1 Å, compared to 2.4 Å between Mn^{2+} at site 1 and this oxygen. The second thallium ion is bound at a site close to site 2 of the Mn^{2+} complexes and is defined by the carboxylate groups of residues

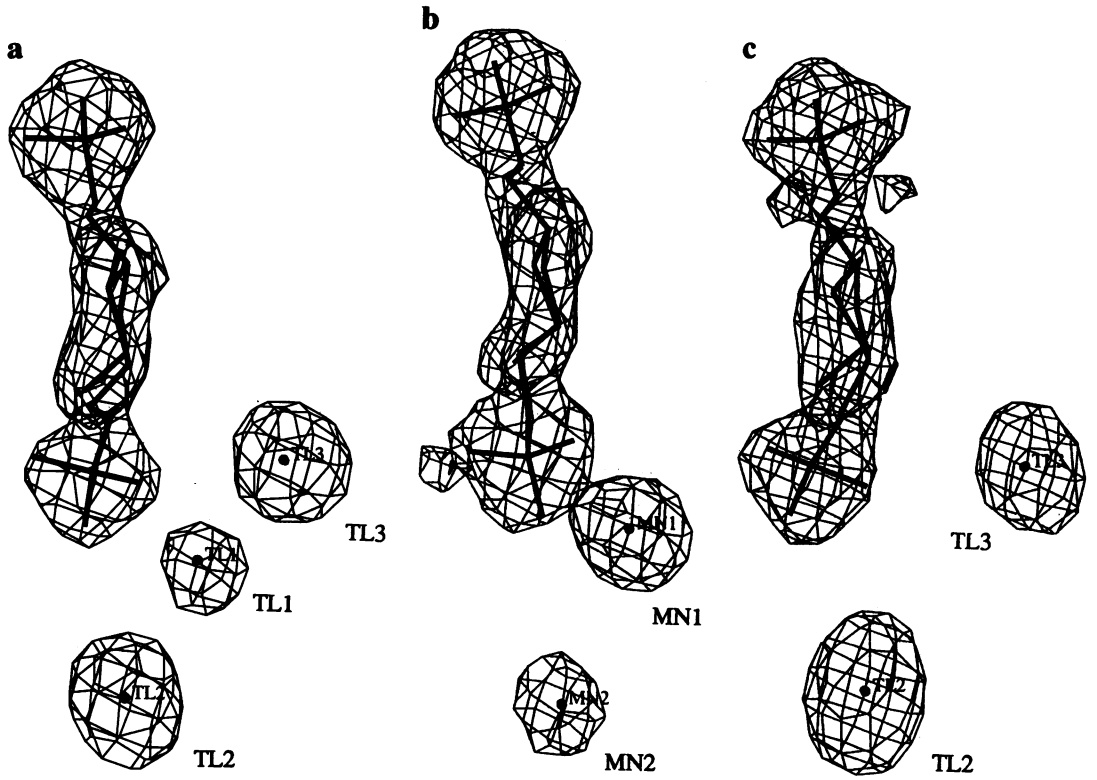

FIG. 1. $F_o - F_c$ density maps calculated by omitting the substrate analogue AhG-1,6-P_2 and the metal ions in the T form Fru-1,6-Pase complexed with Tl^+ ions (*a*), the T form Fru-1,6-Pase complexed with Mn^{2+} ions (*b*), and the T form Fru-1,6-Pase complexed with Tl^+ and Li^+ ions (*c*). The maps are contoured at the 4σ level. Tl^+ sites are referred to (top to bottom) as sites 3, 1, and 2, respectively. Mn^{2+} sites are referred to as sites 1 and 2. The new binding site for thallium ions (site 3) is clearly visible and is 3.4 Å away from Tl^+ at site 1 (*a*). Tl^+ is no longer observed at site 1 when Li^+ ions are present (*c*).

Glu-97, Glu-98, Asp-118, and the carbonyl oxygen of Leu-120. The average distance between these residues and Tl^+ is 2.9 ± 0.1 Å. In the Mn^{2+} complex, Mn^{2+} at site 2 is closer to the carboxylate group of Asp-118 (average distance, 2.4 Å), although similar distances are observed between Mn^{2+} and the other residues. The distance between Tl^+ ions at sites 1 and 2 is 3.9 Å, shorter than the distance previously reported between the two Mn^{2+} ions (4.3 Å) (13).

In the T form structure, the third Tl^+ ion is slightly displaced when compared to its position in the unligated R form–Tl^+ complex, as a consequence of the binding of AhG-1,6-P_2 in the active site. The two Tl^+ ions at sites 3 are 0.5 Å apart when the fructose bisphosphate binding domains of the R and T form enzymes are superimposed. This third site is defined by the carboxylate group of Glu-280 (3.1 Å from Tl^+), the 1-phosphate group of the substrate analogue (the distance between the closest 1-phosphate oxygen and Tl^+ is 3.8 Å), and the guanidinium group of Arg-276 (3.3 Å away) (Fig. 2). This site is occupied not by a divalent metal ion in the Mn^{2+} complex but by the guanidinium group of Arg-276, which has moved away from the 1-phosphate group when Tl^+ ions are present. The distance between Tl^+ at sites 1 and 3 is 3.4 Å (Fig. 3).

Potassium Binding Sites. The overall structures of the T form Fru-1,6-Pase complexed with either Tl^+ or K^+ ions are very similar. Three potassium ions are also identified in the active sites of Fru-1,6-Pase at binding sites corresponding to those described for the thallium complex.

Thallium–Lithium Complex. With only three electrons, lithium ions are nearly invisible in x-ray analysis. In an earlier study, Li^+ ions were not detected in the active site of the T form enzyme cocrystallized in the presence of 35 mM Li_2SO_4 (V.V. and W.N.L., unpublished results). To determine the binding sites of lithium ions, we subsequently soaked a T form crystal grown in the presence of 5 mM Tl_2SO_4 in a buffer containing 5 mM Li_2SO_4. Lithium ions are strong inhibitors of fructose-bisphosphatase; Marcus and Hosey (18) reported an apparent K_i value of 0.3 mM for Li^+ ions in the presence of a saturating concentration of Mn^{2+}. Therefore, in the presence of 5 mM Li_2SO_4, substitutions or reduced occupancies for thallium ions bound at the lithium sites were expected.

Two thallium ions are found in the active site of this complex at metal binding sites 2 and 3. The occupancy factors for these two Tl^+ ions compare well with occupancy factors observed in the complex of the T form with Tl^+ (site 2, 0.65; site 3, 0.5). These observations suggest that lithium ions do not bind to these sites. On the other hand, in the presence of Li^+, Tl^+ at site 1 is no longer present (Fig. 1). Thus, this site is potentially the metal binding site for lithium ions.

DISCUSSION

IMPase and inositol polyphosphate 1-phosphatase share a similar folding with Fru-1,6-Pase despite minimal overall sequence identity (19, 21, 31). These enzymes also require divalent metal cations for catalysis and display a similar behavior toward Li^+ ions. Moreover, these two phosphatases show strong local sequence identity in the metal binding region. Inhibition of IMPase by Li^+ ions is of particular interest, because this enzyme is one of the potential targets for lithium ions used to regulate the phosphatidylinositol cycle in the therapeutic treatment of manic depression. Despite extensive crystallographic studies (20, 32, 33), the binding sites for lithium ions have not been conclusively demonstrated, although it has been suggested, on the basis of detailed kinetic and Tb^{3+} fluorescence quenching studies, that Li^+ ions bind at metal site 2 of IMPase (which corresponds to metal site 1 in Fru-1,6-Pase) and interact with an enzyme–substrate (or intermediate or product) complex (21, 34, 35). Like Fru-1,6-

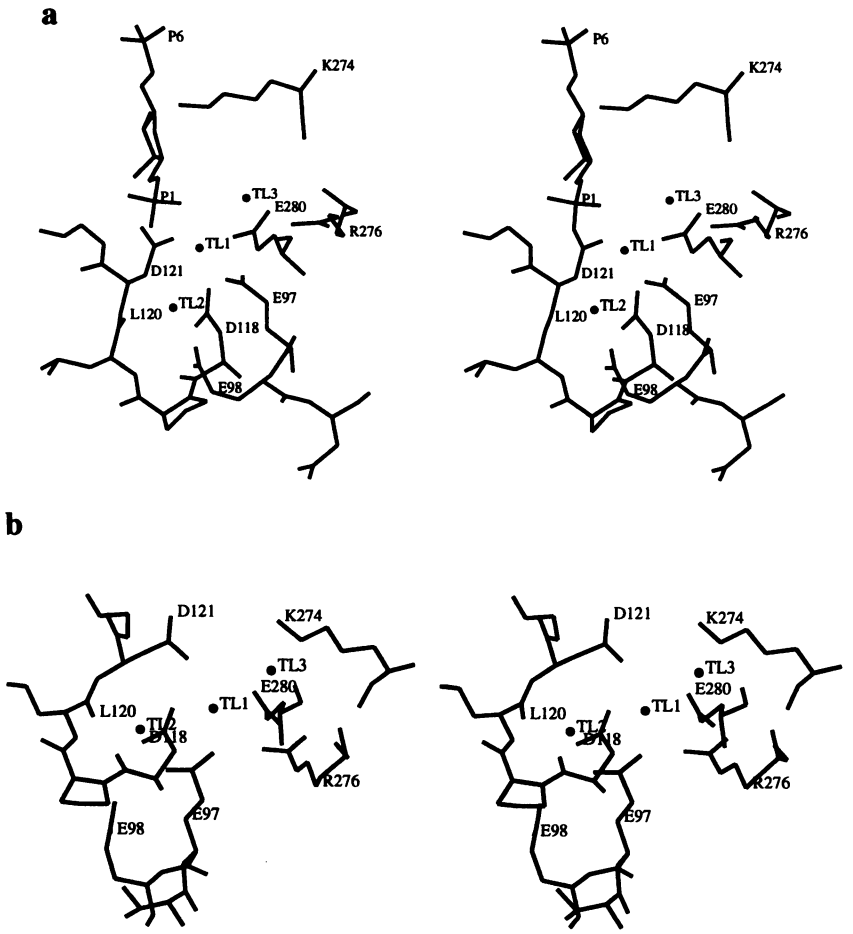

FIG. 2. (*a*) Stereoview of the metal binding region of Fru-1,6-Pase in the T form structure in the presence of AhG-1,6-P_2 and thallium ions. TL1 and TL2 are bound at sites close to the divalent metal sites previously identified. TL3 is bound at a new metal site defined by the 1-phosphate group of AhG-1,6-P_2, Glu-280, and Arg-276. (*b*) Metal binding region of Fru-1,6-Pase in the R form structure. The three thallium ions are similarly labeled. The 6-phosphate and the 1-phosphate groups of the substrate analogue are labeled P6 and P1, respectively.

Pase, IMPase is also activated by K^+ ions (22). However, crystallographic studies of IMPase in the presence of K^+ or Tl^+ ions have not been reported.

We demonstrate that potassium and thallium ions bind at three metal sites located in the active site of Fru-1,6-Pase. Two sites correspond to the divalent metal sites previously identi-

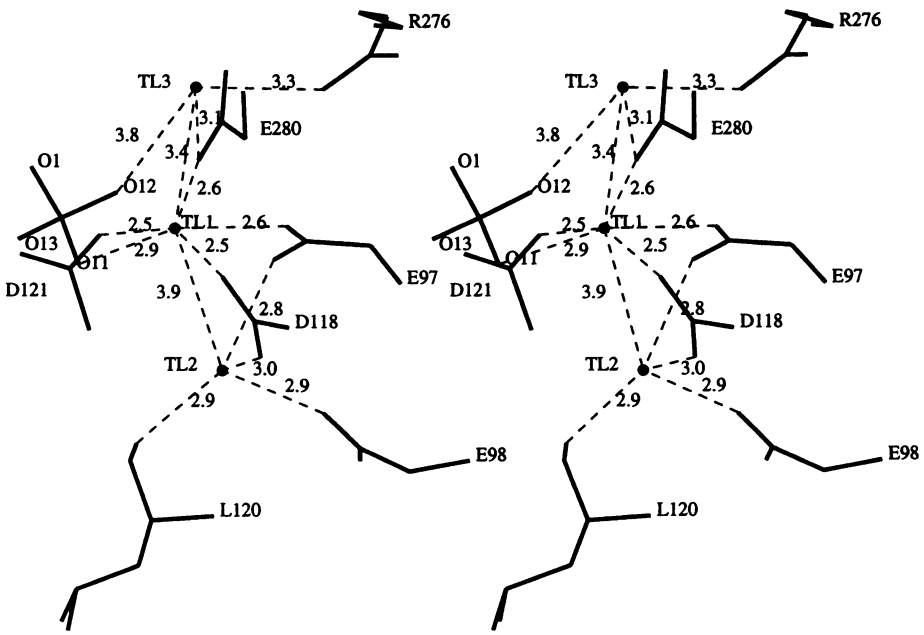

FIG. 3. Schematic stereoview of the three thallium ions and their ligands in the T form Fru-1,6-Pase complexed with AhG-1,6-P_2. Only the 1-phosphate group of AhG-1,6-P_2 is shown. The 1-phosphate oxygens are labeled O11, O12, O13, and O1, respectively. Distances (Å) between thallium ions and their ligands are also indicated.

fied and are likely not occupied by monovalent cations when divalent metals are present. The third Tl^+ or K^+ site, specific to these cations, is 3.4 Å away from site 1 and is defined by Glu-280, Arg-276, and the 1-phosphate group of the substrate analogue.

The identification of this new binding site (site 3) supports a direct role for K^+ or Tl^+ in the catalysis. In the absence of these cations, site 3 is occupied by the guanidinium group of Arg-276. We suggest that Tl^+ or K^+ ions substitute efficiently for Arg-276 in catalysis, resulting in activation of the enzyme. From our previous crystallographic studies, the role of this arginine in catalysis is unclear; the guanidinium group of Arg-276 has been located close (2.8–3.2 Å) to a 1-phosphate oxygen of the substrate analogue in only one of the two monomers present in the asymmetric unit, in either the R or T form crystals [in the second monomer of the Mn^{2+} complexes, the distance is between 3.6 and 4.5 Å (13)]. Mutation of Arg-276 to Met resulted in a significant decrease in the enzyme's activity; the k_{cat} for this mutant was ≈0.67% that of the wild-type enzyme. This mutation causes a total loss of Mg^{2+} cooperativity and changes the kinetic mechanism from rapid-equilibrium random or steady-state ordered Bi Bi to rapid-equilibrium ordered Bi Bi (36). The k_m for Fru-1,6-P_2 was similar to that of the wild-type enzyme (24). One probable role of Arg-276 is to polarize the 1-phosphate group of the substrate, thus helping nucleophilic attack at the phosphorus center.

Only the Tl^+ ion at site 1 was substituted by Li^+, and the occupancy of Tl^+ at site 3 was not significantly affected. Therefore, the hypothesis that inhibition of Fru-1,6-Pase by Li^+ could arise from the binding of these ions to both this new site (site 3) and the adjacent divalent metal site (site 1) is not tenable.

Inhibition of Fru-1,6-Pase very likely occurs upon binding of Li^+ ions at the high-affinity divalent metal site. Detailed kinetic analysis for IMPase suggested that Li^+ binds to a phosphate-bound species after hydrolysis. Another phosphate oxygen derived from solution becomes available for metal binding, and Li^+ may form a tight complex resulting in optimal size and charge for phosphate and protein coordination in the modified metal site, retarding phosphate release (21).

Kinetic investigations of native Fru-1,6-Pase and mutants in which residues defining the metal binding sites have been replaced will provide insights for the action of these monovalent cations. Glu-280 and Arg-276, two critical residues in K^+ and Tl^+ activation, are replaced by an aspartate and a histidine, respectively, in the active site of IMPase. Therefore, despite the similarities of the active sites of both phosphatases, it is not straightforward to suggest a similar mechanism of activation. Crystallographic investigations of IMPase complexed with Tl^+ or K^+ will be of great interest in understanding monovalent cation effects on this phosphatase.

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