Crystal structures of the active site mutant (Arg-243 → Ala) in the T and R allosteric states of pig kidney fructose-1,6-bisphosphatase expressed in *Escherichia coli*

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Abstract

The active site of pig kidney fructose-1,6-bisphosphatase (EC 3.1.3.11) is shared between subunits, Arg-243 of one chain interacting with fructose-1,6-bisphosphate or fructose-2,6-bisphosphate in the active site of an adjacent chain. In this study, we present the X-ray structures of the mutant version of the enzyme with Arg-243 replaced by alanine, crystallized in both T and R allosteric states. Kinetic characteristics of the altered enzyme showed the magnesium binding and inhibition by AMP differed slightly; affinity for the substrate fructose-1,6-bisphosphate was reduced 10-fold and affinity for the inhibitor fructose-2,6-bisphosphate was reduced 1,000-fold (Giroux E, Williams MK, Kantrowitz ER, 1994, *J Biol Chem* 269:31404–31409). The X-ray structures show no major changes in the organization of the active site compared with wild-type enzyme, and the structures confirm predictions of molecular dynamics simulations involving Lys-269 and Lys-274. Comparison of two independent models of the T form structures have revealed small but significant changes in the conformation of the bound AMP molecules and small reorganization of the active site correlated with the presence of the inhibitor. The differences in kinetic properties of the mutant enzyme indicate the key importance of Arg-243 in the function of fructose-1,6-bisphosphatase. Calculations using the X-ray structures of the Arg-243 → Ala enzyme suggest that the role of Arg-243 in the wild-type enzyme is predominantly electrostatic in nature.

Keywords: metalloenzymes; mutagenesis; protein structure–function; site-specific; X-ray diffraction

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Abbreviations: Fru-1,6- P_2 ase, fructose-1,6-bisphosphatase; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; Arg-243 → Ala, the mutant pig kidney fructose-1,6-bisphosphatase with arginine at position 243 replaced by alanine; side-chain conformation is represented by a symbol (t, +, –), which is equivalent to (180 degrees, +60 degrees, –60 degrees) RMSD, RMS displacement;

Fructose-1,6-bisphosphatase (E.C. 3.1.3.11) is a tetrameric enzyme playing an important role in metabolic control of gluconeogenesis (Fig. 1). Each identical subunit has 337 amino acid residues and a molecular weight of about 36,500 (Marcus et al., 1982; Williams & Kantrowitz, 1992). Mammalian enzymes (liver and kidney) are more than 85% homologous in amino acid sequence (El-Maghrabi et al., 1988). The physiologic regulators AMP and Fru-2,6- P_2 negatively modulate enzyme activity and, in reciprocal fashion, positively affect the activity of phosphofructokinase, a control point in glycolysis (for a review, see Pilks & Claus, 1991).

Studies of the mechanism of regulation of Fru-1,6- P_2 ase by the effectors, and of the catalytic mechanism of hydrolysis of Fru-1,6- P_2 , have been extended to the structural level by the availability of crystal structures for pig kidney Fru-1,6- P_2 ase (Ke et al., 1989b) and complexes of this enzyme with hydrolytic product Fru-6-P (Ke et al., 1991b), with inhibitor AMP (Ke et al.,

$$R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_{\text{mean}} - I_i|}{\sum_{hkl} \sum_i I_i}; \quad R = \frac{\sum |F_{\text{Obs}} - F_{\text{Calc}}|}{\sum F_{\text{Obs}}}$$

Fig. 1. A: Schematic view of the tetramer with the AMP and F-6-P domains indicated. The active site is denoted by "O," the AMP affecter binding site by "X," and the metal ions by a diamond at the domain interface. **B:** Ionic interactions involving Arg-243 and Lys-274 at an active site of pig kidney Fru-1,6- P_2 ase, based on the crystal structure of the complex with inhibitor Fru-2,6- P_2 bound. The coordinates used for this figure were provided by V. Villeret and W.N. Lipscomb. Arg-243 from one subunit (in thicker line) interacts with the 6'-phosphate of the inhibitor, whereas Lys-274 of the subunit forming the active site pocket interacts with the 6'-phosphate and the furanose ring of the inhibitor. Similar interactions are also observed with the substrate Fru-1,6- P_2 (Zhang et al., 1993).

1991a) and with inhibitor Fru-2,6- P_2 (Ke et al., 1989a), among others. The product complex is taken as an example of the allosteric R state, whereas the AMP complex represents the T state (Liang et al., 1993).

Despite the fact that the enzyme has been cloned from several different sources, including pig kidney (Williams & Kantrowitz, 1992), pig liver (Burton et al., 1993), rat liver (El-Maghrabi et al., 1988; El-Maghrabi & Pilkis, 1991), and human liver (El-Maghrabi et al., 1993), the X-ray structures are known only for three different enzymes. These are from human liver (Gidh-Jain et al., 1994), spinach chloroplast (Villeret et al., 1995b), and pig kidney (Ke et al., 1989b). Analysis of the crystal structure of pig kidney Fru-1,6- P_2 ase, which was first to be solved, has suggested several amino acid residues that may have functional importance. Site-specific mutagenesis has been used to test whether these residues are actually important for the function of the enzyme. Residues such as Lys-274 (El-Maghrabi et al., 1992), using the numbering system of the pig kidney enzyme, which interact with substrate at the active sites, Glu-97, Glu-280, Asp-118, Asp-121, and Arg-276 (Chen et al., 1993; El-Maghrabi et al., 1993; Zhang & Fromm, 1995) at the metal-binding sites, and Tyr-113, Glu-29, Thr-31, and Arg-22 (Chen et al., 1994; Gidh-Jain et al., 1994; Lu et al., 1995) at the AMP-binding sites have been investigated.

The X-ray structure of the pig kidney enzyme shows that Arg-243 from one subunit interacts with the 6-phosphate portion of sugar phosphates (Ke et al., 1989a) in the active site of an adjacent subunit. To assess the importance of this intersubunit interaction for catalysis or inhibition of Fru-1,6- P_2 ase, site-specific mutagenesis was used to replace Arg-243 with alanine in the cloned pig kidney enzyme (Williams & Kantrowitz, 1992).

Hydrolysis of substrate carried out by the wild-type Fru-1,6- P_2 ase displays hyperbolic kinetics at neutral pH, with partial inhibition at high substrate concentrations, whereas plots of reaction velocity versus cofactor magnesium concentration are sigmoidal (Nimmo & Tipton, 1975b). Inhibition by AMP is cooperative, with a Hill coefficient near 2 (Taketa & Pogell, 1965; Nimmo & Tipton, 1975a), whereas Fru-2,6- P_2 binds to the active sites of the enzyme (Ke et al., 1989a) in competition with the substrate Fru-1,6- P_2 (Pilkis et al., 1981). The AMP binding site on each subunit is 28 Å distant from the active site (Ke et al., 1991a) and the metal-binding sites are located between the Fru-1,6- P_2 and AMP domains (Ke et al., 1990).

Kinetic characteristics of the altered enzyme (Arg-243 → Ala) showed that magnesium binding and inhibition by AMP differed less than one order of magnitude; affinity for the substrate Fru-1,6- P_2 was reduced 10-fold, whereas affinity for the inhib-

itor Fru-2,6-P₂ was reduced 1,000-fold. To further our understanding of the kinetic properties of the Arg-243 → Ala enzyme and to correlate the altered kinetic properties of the mutant enzyme with specific structural features, we determined the three-dimensional X-ray structure of the Arg-243 → Ala enzyme in both the R and T states.

Results

Quality and description of the structures

The structure refinements of the T state models were initiated by extracting the atomic data (1frp) from the Protein Data Bank (Bernstein et al., 1977). The structure used was a model of the T state refined at 2 Å resolution to an *R*-factor of 18.3% (Xue et al., 1994) (Fig. 1B) with Arg-243 replaced with alanine. Because two complete data sets for the T state structures from different crystals were collected (Table 1), two atomic models were refined independently. Both models of the Arg-243 → Ala Fru-1,6-P₂ase in the T state refined well to final *R*-factors of 19.8% and 19.6% for the first and second crystals, respectively, with excellent geometries (Table 2). Slightly higher weights (1.0 *wa*) than recommended by Brünger (1992) (~0.5 *wa* as calculated in CHECK) were used to allow for the fragments with weaker densities to refine well. The quality of the structures were determined using the program PROCHECK (Laskowski et al., 1993). All of the statistics indicate well refined structures equal to or better than other structures at similar resolution. The backbone torsional angles were well within allowed regions of the Ramachandran plot (not shown) and side-chain torsional angles were tightly clustered around allowed conformers. The final refinement statistics are presented in Table 2. The Luzzati plots (Luzzati, 1952) for the T state structures indicated a positional error of 0.275 Å and 0.25 Å for the first and the second models, respectively.

The structures show little divergence from other T state structures of the wild-type enzyme (Ke et al., 1990, 1991a; Xue et al., 1994). The RMS difference (RMSD) variation between the final models and the initial wild-type structure were 0.37 Å and 0.39 Å for the backbone atoms and 0.98 Å and 0.99 Å for the side-chain atoms, respectively. Both structures had similar conformation of the backbone, with the exception of disordered

fragments 1–6 and 62–69. The RMSD between the two T state structures was 0.21 Å (Fig. 2), similar in magnitude to the positional error estimated from the Luzzati plots (0.25 Å).

The Fru-1,6-P₂ase structure contains a dimer in the asymmetric part of the unit cell and forms a tetramer across the crystallographic twofold axis. As described previously (Ke et al., 1991a), each subunit forms well-defined secondary structure elements, 9 α -helices and 11 β -strands in two distinct β -sheets. In the individual subunits, two structural/functional domains can be distinguished. The allosteric AMP domain comprises three helices, which form an interface with other subunits, and a six-stranded antiparallel β -sheet with two short connecting helices. The second Fru-1,6-P₂ domain contains four helices and a five-stranded antiparallel β -sheet. The metal binding sites are located between the two structural domains (Fig. 1A). The only modification to this already described structure is that, in accord with the amended sequence, 337 amino acids were modeled in each subunit including N- and C-terminal fragments.

In both T form structures, the electron density clearly indicated that AMP and Fru-6-P were bound within each subunit. The metal binding site was unoccupied, but differences between the two independent subunits revealed that, despite the lack of clear difference densities defining the metal ion, the C2 subunit may have some residual occupancy of a metal ion (most likely Mg²⁺). The details of the binding of effectors are presented below.

Despite the lack of the crucial active site residue (Arg-243), which interbridges the two subunits and participates in substrate/inhibitor binding, there were no major changes in the organization of either the secondary, tertiary, or the quaternary structure of the enzyme. The plot of observed RMSDs between the wild-type enzyme structure and the two T state models is presented in Figure 2.

The refinement of the R state model at 2.75-Å resolution was initiated from the Protein Data Bank file (code 5fbp) refined at 2.1-Å resolution (Ke et al., 1991b). The model refined well to the crystallographic *R*-factor of 22.3% with reasonable geometry. For the enzyme in the R state, the initial structure showed much more disagreement with the data, the initial *R*-factor was 43.3%, compared with the structures of the enzyme in the T state. This observation has been reinforced by the final structure. The model of the mutant enzyme showed much larger de-

Table 1. Summary of the data collection for the Fru-1,6-P₂ase Arg-243 → Ala mutant enzyme

Enzyme form	Space group	<i>d</i> _{max} (Å)	s(I) I ^a	Reflections unique	Completeness	Redundancy	Unit cell (Å)	Final <i>R</i> _{merge} (%)
T-form first crystal	P2 ₁ 2 ₁ 2	2.2	1.05	36,660	86%	2.1	<i>a</i> = 61.20 <i>b</i> = 166.75 <i>c</i> = 79.84	9.65
T-form second crystal	P2 ₁ 2 ₁ 2	2.05	1.0	47,244	88%	2.3	<i>a</i> = 61.06 <i>b</i> = 166.78 <i>c</i> = 79.84	7.95
R-form	P3 ₂ 21	2.75	1.0	16,825	97%	3.6	<i>a</i> = <i>b</i> = 131.31 <i>c</i> = 66.40	13.77

^a Signal to noise ratio in the highest resolution shell.

Table 2. Refinement statistics for the models of Fru-1,6-P₂ase Arg-243 → Ala mutant enzyme

Enzyme form	Final <i>R</i> -factor	<i>d</i> _{min} (Å)	Ligands (occupancy)	Average B (Å ²)		No. of water molecules	RMSDs	
				backbone	side chain		Bond length (Å)	Bond angles (°)
T-form first crystal	0.198	2.2	2 × Fru-6- <i>P</i> (0.8)	F6P	AMP	118	0.011	1.78
T-form second crystal	0.196	2.05	2 × Fru-6- <i>P</i> (1.0)	F6P	AMP	180	0.011	1.70
R-form	0.223	2.75	2 × Fru-6- <i>P</i> (1.0)	F6P	AMP	59	0.012	2.0

parture from the initial model with an RMSD of 1.05 Å for backbone atoms and 1.54 Å for side chains. The structure showed, however, the same kind of transformation from the T to R state with an ~15 degree rotation between the two dimers, as seen earlier (Ke et al., 1991a) (Kinemage 1). Because of much weaker data, the degree of change is difficult to evaluate. Nevertheless, it is quite clear that the effector AMP site is unoccupied (Fig. 3) and the inhibitor Fru-6-*P* is bound at both active sites.

Active site and binding of Fru-6-*P*

In order to determine possible alterations in the active site of Fru-1,6-*P*₂ase due to the replacement of Arg-243 by Ala, we have investigated the mutant enzyme in the presence of the inhibitor Fru-6-*P*. When the diffraction data were collected for the first crystal in the T state, we noticed that, despite the use of size-exclusion chromatography and dialysis during the purification, the enzyme was in the T state as judged by the space

Fig. 2. Plot of the RMS deviation calculated for the backbone atoms between the C1 subunit of the second 2.05-Å model of the T state and the C1 subunit of initial model of the T state (1frp) (solid line), C1 subunit of the first T state model (2.2 Å, in broken line) and the C2 subunit of the second model (small dashed line).

Fig. 3. AMP binding site in the model of the R state structure. The $2F_o - F_c$ electron density (thin line) is contoured at the 1.2σ level and $F_o - F_c$ electron density at 2.4σ (thick broken line). No substantial residual density indicates binding of AMP.

group (P2₁2₁2) and cell dimensions. Furthermore, when the refinement was initiated, we could clearly see in the omit maps at the 3σ level two tightly bound AMP molecules, one in each subunit, in addition to Fru-6-P bound at the active site (Kine-mage 2). In the final structure, AMP refined to occupancy 1.0, whereas the inhibitor (Fru-6-P) refined to occupancy 0.8 in both subunits.

The data collection on the second crystal resulted in a better data set (~47,000 reflections, $R_{\text{merge}} = 7.8\%$). Unexpectedly, when a second model was refined against this data set, the inhibitor, Fru-6-P, refined to full occupancy at both active sites. Most of the active site residues were clearly defined in the $2F_o - F_c$ maps (Fig. 4). Furthermore, there was no detectable

difference density corresponding to the presence of the Arg-243 at the active site in either of two T state structures. Despite the higher resolution and lower R -factor for the second model, several active site residues such as Tyr-256, Ser-124, Asn-125, Lys-268, and Lys-269 had weaker densities, suggesting a more dynamic state of the enzyme. Mobility of these residues is consistent with the reported changes in conformations for these residues in the transition from the T to the R state (Xue et al., 1994).

The Fru-6-P inhibitor bound in the active site is clearly visible in the electron density maps and its positioning is unambiguous. The phosphate group of the inhibitor is held by numerous hydrogen bonds to Tyr-215, Asn-212, Tyr-244, Tyr-264, Lys-274,

Fig. 4. Active site of Arg-243 → Ala enzyme in the second T state structure. The electron densities are contoured at the same level as in Figure 3. No residual density is observed at the Arg-243 side chain, indicating no contamination with the wild-type enzyme.

and the O3 oxygen of the furanose ring to the amide nitrogen of Met-248 and carboxyl group of Asp-121 in the very same manner as in the wild-type enzyme (Ke et al., 1990). The absence of Arg-243 is compensated by additional hydrogen bonds to solvent molecules.

In the R state form, the binding of Fru-6-P is very similar to the T form, and to that found in the native wild-type enzyme (Ke et al., 1991a). The interactions of the enzyme to Fru-6-P in the crystal structures presented here are summarized in Table 3.

Allosteric site and AMP binding

The surprising result is that enzyme from the same preparation yielded two different conformational states of the AMP depending on the occupancy of the inhibitor molecule Fru-6-P in the active site, and that the less ordered state of the enzyme was associated with full occupancy.

As mentioned above, two AMP molecules, one at each subunit, were detected in both models of the T state of the Arg-243 → Ala enzyme. In both structures, the AMP molecules refined to full occupancy. The phosphate groups have well-defined interactions with backbone nitrogens of residues Thr-27, Gly-28, Glu-29, and Met-30, and of side chains of Thr-27, Lys-112, and Tyr-113, which also interacts with O3' of the ribose ring (Table 4). Both ribose oxygens are within hydrogen bonding distance of guanidinium nitrogens of Arg-140. In the first crystal, the conformation of the adenosine ring is in the *syn* conformation, with two distinct hydrogen bonds to the side chain of Thr-31 (N1) and the carbonyl oxygen of Val-17 (N6) (Fig. 5; Kinemage 3). The *syn* conformation was first observed in the 2.5 Å structure of the native enzyme complexed with Fru-6-P and AMP (Ke et al., 1990). This conformation was confirmed by inspection of the omit maps and analysis of the distribution of temperature factors of the AMP molecule. In solution, the *syn* conformation is much less frequent and, as expected, energy minimization calculations have shown that it has higher energy than the molecule in the *anti* conformation (Saenger, 1984).

The refinement of the second T state structure showed that the adenosine ring of the AMP molecule bound to the C2 subunit was in the *anti* conformation (Fig. 6). In this conformation,

Table 3. Hydrogen bonding pattern for Fru-6-P

	First (2.2 Å) T state model		Second (2.05 Å) T state model	
	C1	C2	C1	C2
O21-N212 ND2	2.87	2.87	3.02	2.90
O21-Y244 OH	3.37	3.03	2.68	2.53
O22-WAT63			3.24	
O22-WAT70				2.87
O23-Y264 OH	3.03	3.82	2.86	2.74
O23-Y264 OH	3.03	3.82	2.86	2.74
O6-K274 NZ	2.94	3.34	2.63	2.81
O5-K274 NZ	2.87	3.16	3.08	3.31
O3-M248 N	2.75	2.94	2.63	2.81
O3-D121 OD2			2.73	3.03
O1-R276 NH2			3.03	3.31

Table 4. Hydrogen bonding pattern for AMP

	First (2.2 Å) T state model		Second (2.05 Å) T state model	
	C1	C2	C1	C2
O-K112 NZ	2.60	2.52	2.83	2.57
O-Y113 OH	3.17	3.12	2.68	2.81
O-M30 N	3.72	2.90	2.75	2.68
OL-T27 OG1	2.63	2.84	2.88	2.78
OL-T27 N	2.75	2.91	2.75	2.91
OR-M30 N	3.24	3.12	2.90	3.04
OR-E29 N	3.12	3.41	2.78	2.68
OR-G28 N	3.28	2.96	3.21	2.92
O5-Y113 OH	2.83	2.76	2.83	2.76
O3'-Y113 OH	2.49	2.35	2.52	2.47
O3'-R140 NE	2.87	2.88	3.39	3.51
O3'-R140 NH1	3.08	3.04	3.20	2.96
O2'-R140 NH1	3.08	3.04	2.92	3.43
N6-V17 O	3.05	2.90	3.58	2.92
N6-T31 OG1				3.41
N1-T31 OG1	2.82	2.93	3.42	

the ring loses one of the hydrogen bonds to N1 and Oγ1 of Thr-31 is now bonded to the adenosine N6 nitrogen. This mode of interaction seems to be less stable than the *syn* conformation found in the first crystal. It appears, however, to be preferred, as might be inferred from the equilibration time of the crystals; the second data collection performed on the crystal equilibrated for a much longer period of time with crystallization buffer, which resulted in full occupancy of Fru-6-P. The details of the AMP interactions with the enzyme in both crystals are summarized in Table 4.

Differences between the two T state models: Conformation of the active site residues in the C1 and C2 subunits and presence of metal ions

Two independent determinations of the T state structure gave us the opportunity to investigate the differences between the individual subunits in a more quantitative way. They also provided the reference point for observed changes correlated to the occupancy of the inhibitor. In the first 2.2 Å, T state model, residues 119–121 have relatively low temperature ($\sim 22 \text{ Å}^2$) factors and Tyr-258 ($B = 25 \text{ Å}^2$) has a very well-defined density and hydrogen bonding pattern (Fig. 7). In the second structure, the same residues exhibit higher temperature factors ($\sim 28 \text{ Å}^2$) and the electron density for Tyr-258 is much weaker ($B = 70 \text{ Å}^2$). This would suggest that the protein as determined from the second crystal is conformationally close to the T state, but its second structure represents a transition toward the R state conformation. It is noteworthy that the residues that take part in the observed transformation from the T to R state are identifiable from the comparison of the two T state structures.

Although the active sites of both independent subunits (C1 and C2) are very similar, some notable differences are observed. The same residues (see above) appear to be more mobile between the subunits and they show much larger RMSD variation than other active site residues (see Fig. 2).

Fig. 5. AMP binding site with the AMP bound in the second T state structure of the Arg-243 → Ala enzyme. The view is similar to that in Figure 3. The $2F_o - F_c$ electron density map covering the AMP molecule is contoured at the 1.2σ level.

Another interesting residue is Arg-276. This residue has distinctly different conformations in the C1 and C2 subunits. In the C2 subunit, Arg-276 extends into solvent in the $(-, +, +, t, t)$ conformation and has a higher average temperature factor of

$50.7 \text{ \AA}^2$, whereas in the C1 subunit, it points toward the bound Fru-6-P in the $(-, -, t, +, t)$ conformation and has a lower temperature factor of $33.27 \text{ \AA}^2$. In both T state structures, the observed conformational variability between the subunits could be

Fig. 6. **A:** Superimposition of the C1 and C2 AMP binding site with AMP in *syn* and *anti* conformations as refined in the second T state structure of the Arg-243 → Ala enzyme. In the first T state structure, both AMP molecules are in the *syn* conformations. The labeling scheme reflects the subunit at which the AMP molecule was bound and refined. The AMP molecule in **(B)** the *syn* and **(C)** the *anti* conformation bound at the C1 and C2 subunit, respectively, covered with the $2F_o - F_c$ electron density covered at 1.3σ level. The temperature factors (in $\text{\AA}^2$) are shown next to the atom label.

Fig. 7. Tyr-258 covered with the $2F_o - F_c$ electron density contoured at 1.4σ level. **A:** The first T state structure. **B:** The second T state structure. Much weaker density indicates the dynamic state of this tyrosine side chain, leading to the reorganization seen in the R state form of the enzyme (broken lines).

correlated with residual density at the metal binding site. The conformation of the Arg-276 in the C1 subunit can be seen in Figure 4. In the C1 subunit, the metal binding site is empty in both crystals, but, in the C2 subunit, a small residual density was detected. This density might be interpreted as a metal ion at low occupancy (most likely Mg^{2+} for its low atomic mass). Recently, Arg-276 has been mutated (Zhang & Fromm, 1995) and the authors concluded that this residue plays a crucial role in determining the cooperativity and affinity for the metal ions (Zhang & Fromm, 1995). The difference in conformation observed in our structure might be a structural illustration of this ascribed role of Arg-276. In the R form, the conformation of this arginine residue is like that observed in the C1 subunit of the T state structure.

Modeling of fragments 1–8, 62–70, and 355–357 in the T state structures

The electron density maps for fragments 1–8, 62–70, and 355–357 were very weak, as was observed for the wild-type enzyme, and initially these fragments were not modeled. When the R -factors reached $\sim 22\%$ and the quality of the maps improved, the decision was made to determine the most probable conformations for these disordered fragments. In addition to the back-

bone stereochemistry, three guiding principles were used: the presence of the positive and negative difference maps on different contouring levels, the magnitude of temperature factors, and the R_{free} . From the R_{free} standpoint, modeling of these residues is insignificant, but, from the point of view of the difference density, we have obtained much flatter difference maps without outstanding positive or negative peaks and, additionally, the temperature factors of these fragments are not excessively high ($\sim 100 \text{ \AA}^2$). After removing those fragments, relatively well defined, positive difference densities were obtained, indicating that, even though the fragments are disordered, they attain relatively few preferred conformations.

The final two residues (Ala-336 and Lys-337) that were not included in previous structure determinations were modeled and refined. Both amino acids had elevated temperature factors, but the electron density maps unequivocally supported the modeling (Fig. 8). In the R state structure, all the disordered fragments (1–8, 62–69, and 335–337) were modeled by analogy to the T state models. The initial conformations were taken from the T state structures and refined. Although the R -factor of the final model was very similar, the electron density improved substantially. The difference electron density became much flatter and the $2F_o - F_c$ maps improved substantially and covered 70% of newly modeled amino acids.

Fig. 8. $2F_o - F_c$ electron density maps covering the newly refined Ala-336 and Lys-337 residues in the second T state structure of the Arg-243 $\rightarrow$ Ala enzyme. **A:** C1 subunit. **B:** C2 subunit. The map is contoured at 1σ level.

Verifying predictions of the molecular dynamics

Molecular dynamics calculations were used to determine possible alterations in the active site of Fru-1,6- P_2 ase due to the replacement of Arg-243 by Ala. The predicted structural changes were described in a previous report (see Fig. 6 in Giroux et al., 1994). Analysis of the molecular dynamics simulations suggested (see Fig. 6 in Giroux et al., 1994) that replacement of arginine by alanine at position 243 would result in substantial alteration in the position of inhibitor Fru-2,6- P_2 in the active site of Fru-1,6- P_2 ase. Orientation of the substrate Fru-1,6- P_2 was not altered markedly and positions of the side chains of Lys-269 and Lys-274 were shifted in the mutant enzyme. However, in the Arg-243 $\rightarrow$ Ala molecular dynamics simulations, Lys-274 interacted strongly with both the 2'- and 6'-phosphates, and Lys-269 interacted with the 6'-phosphate group of Fru-2,6- P_2 . For the substrate complex on the other hand, dynamics calculations of the wild-type enzyme indicated interactions of Lys-274 with the 1'-phosphate and interactions of both Arg-243 and Lys-269 with the 6'-phosphate. Both lysine interactions were maintained in the Arg-243 $\rightarrow$ Ala mutant simulations of the substrate complex.

We have evaluated these predictions and compared them with the determined X-ray structures. Molecular dynamics predicted correctly both the position of Lys-274 and only a minor change in position of the inhibitor Fru-6- P . The 6'-phosphate group was

in the same position as in the wild-type enzyme, which would suggest that the molecular dynamics did not predict correctly this structural detail. The predicted changes for Fru-2,6- P_2 were difficult to verify because the affinity for the inhibitor dropped by 1,000-fold and it was not possible to obtain crystals of the mutant enzyme with Fru-2,6- P_2 bound.

Electrostatic calculations aimed at explaining the kinetic results

In order to evaluate the importance of Arg-243 for substrate and inhibitor binding and to understand better the difference between affinities of Fru-6- P and Fru-2,6- P_2 , the electrostatic field acting on the charged phosphate groups of those moieties have been calculated and visualized. The electric field lines and vectors were superimposed and visualized together with the molecular surface of the molecule in the native as well in the mutant enzyme. The calculations indicated that removal of the Arg-243 guanidinium group from the shared active site changed the direction and the magnitude of the electrostatic field in the binding pocket.

The vector of the electric field positioned at the O1' phosphate group did not change significantly either in the direction or magnitude in the mutant enzyme. In contrast, the electric field vec-

tor positioned at the O6' phosphate group changed direction by more than 60 degrees, and in the mutant enzyme, it is directed almost perpendicular to the protein surface. The strength of the field is slightly increased, indicating a weaker binding for the negatively charged phosphate group. The field lines were more dispersed in the mutant, indicating a less defined binding path for both substrates and inhibitors having O6' phosphate groups. The most pronounced change was observed at the O2' site of the inhibitor. Although there is little change in the direction of the electric field, there is a significant difference in its magnitude at the phosphate group O2' position.

Discussion

Arg-243 is the only active site residue of Fru-1,6- P_2 ase, which interbridges the subunits (see Fig. 1B). Each of the four active sites of the tetrameric enzyme being constituted with an Arg-243 from a neighboring polypeptide chain in a dimer (Ke et al., 1989a). Because Tyr-244 is another active site residue that forms a backbone hydrogen bond to its dimer mate, the two Arg-243 residues from both subunits are spatially separated only by the length of two peptide bonds. All other significant components of each active site, and of the AMP-binding region as well, derive from a continuous polypeptide chain. X-ray crystallographic studies of pig kidney Fru-1,6- P_2 ase indicate that Arg-243 interacts principally with the 6-phosphate group of the substrate Fru-1,6- P_2 and the inhibitor Fru-2,6- P_2 (Ke et al., 1989a). Such studies had led to the prediction that replacement of Arg-243 by Ala would destabilize 6-phosphate binding and reduce affinity for substrate (Ke et al., 1991b). Functional data obtained via site-specific mutagenesis were used to investigate this prediction.

Active sites in Fru-1,6- P_2 ase are shared between subunits

The removal of the side chain carrying the guanidinium group at position 243 of pig kidney Fru-1,6- P_2 ase resulted in a func-

tional, but impaired enzyme (Giroux et al., 1994). This mutation at the active site altered the affinity for both magnesium ion and for the inhibitor AMP, the binding regions for which are distant from the substrate binding site. Substrate affinity, and particularly, affinity for the competitive inhibitor Fru-2,6- P_2 , were decreased more significantly.

The X-ray study conducted on two crystals of the Arg-243 → Ala enzyme in the T state showed that there is no major reorganization of the active site as might be suggested by the kinetic data. Some structural details, such as reorganization of the 120s loop and rotation of Tyr-258, might shed light on the conformational changes at the active site when the inhibitor Fru-2,6- P_2 binds. Two binding modes of the allosteric effector AMP were observed, which might correlate with the observed synergism of the inhibitors bound at the active site (Fru-6- P) (Liu & Fromm, 1988). It would be interesting to study the differences in affinity for AMP in the presence of other inhibitors in order to investigate the importance of these two conformations.

The resolution and quality of the data for the protein crystal in the R state made the detailed analysis less reliable. However, it showed a similar reorganization of the enzyme as in the wild-type enzyme, involving an ~15 degree rotation of the subunits (Ke et al., 1991b). AMP was not detected, suggesting that helices H1 and H2 underwent a reorganization similar to that in the wild-type enzyme (Ke et al., 1991a).

Previous molecular dynamics predictions (Giroux et al., 1994) were largely but not completely confirmed by the crystal structure. Modeling of interactions at the active site suggested an altered orientation for docked Fru-2,6- P_2 inhibitor and conformational changes of crucial residues (Lys-274) in the mutant enzyme. The conformation of Lys-274, Lys-269, and the location of the Fru-6- P were found to be similar to those predicted.

Electrostatic calculations on the native as well as on the mutant enzyme structures using DELPHI (Gilson & Honig, 1987a, 1987b) as implemented in GRASP (Nicholls, 1992) indicated changes of the electric field at the phosphate binding site (Fig. 9).

Fig. 9. Active site pocket with bound inhibitors Fru-6- P and 2,5-anhydro-D-glucitol-1,6-bisphosphate (AHG) superimposed (Villeret et al., 1995a). The transparent molecular surface is shown with the potential field mapped onto it in red (negative) and blue (positive potential). Lines and vectors of the electrostatic field are also shown for the mutant Arg-243 → Ala as well for the wild-type enzyme as calculated at the three phosphate group positions. Electric field lines for the mutant are in yellow and vectors in blue. The field for the native enzyme is represented by the green lines and maroon vectors. The picture was created with the program GRASP. The electric field calculated with the program DELPHI from the coordinates of the refined model of the second T state structure with and without Arg-243 present.

The direction of electric field, as well as its magnitude at the phosphate binding site, changed. This would suggest that the role of the side-chain group of Arg-243 is mostly electrostatic. The change in electrostatic field should mostly influence binding of the inhibitor Fru-2,6-*P*₂, which has two phosphate groups (O2' and O6'). These two groups were the ones most affected by the removal of guanidinium group of Arg-243.

The qualitative electrostatic picture suggests a likely explanation for the observed lowering of binding affinities for Fru-6-*P*, Fru-1,6-*P*₂, and also for the significantly lower affinity (~1,000-fold) for Fru-2,6-*P*₂. This functional understanding of kinetic results could only be obtained by directly correlating the functional properties of the mutant enzyme with the structures of not only the wild-type enzyme, but of the structure of the mutant enzyme as well.

Materials and methods

Construction of wild-type and mutant enzyme expression systems and site-specific mutagenesis

Construction of the cDNA library and cloning of the Fru-1,6-*P*₂ase cDNA (Williams & Kantrowitz, 1992), the procedure for creating the Arg-243 → Ala Fru-1,6-*P*₂ase by site-specific mutagenesis, and biochemical characterization of the Arg-243 → Ala enzyme (Giroux et al., 1994) have been described previously. In order to express the Arg-243 → Ala pig kidney Fru-1,6-*P*₂ase, phagemid pEK220 (Giroux et al., 1994) was transformed into *Escherichia coli* strain EK1601. *E. coli* strain EK1601 has a deletion in the chromosomal *fbp* gene and can be induced to produce T7 RNA polymerase (Giroux et al., 1994). In this manner, it was impossible to contaminate the pig kidney Fru-1,6-*P*₂ase expressed from the plasmid with *E. coli* Fru-1,6-*P*₂ase expressed from the chromosome.

Enzyme purification

Bacteria were cultured with vigorous agitation at 37 °C in M9 medium supplemented with 0.5% casamino acids and ampicillin at 100 µg per mL. Induction of T7 RNA polymerase was initiated by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (US Biochemical). After further cultivation for 16–22 h, cells were harvested by centrifugation, broken open by sonication, and purified as described previously (Giroux et al., 1994). More than 40 mg of the Arg-243 → Ala enzyme was obtained from 10 L culture and concentrated to ~15 mg/mL. The enzyme was dialyzed before crystallization against 50 mM Tris buffer, pH 7.5, containing 1 mM MgCl and 0.5 mM EDTA.

Crystallization

Concentrated protein was crystallized by the hanging drop vapor diffusion method in the conditions established in our laboratory, as well as by the dialysis method (Ke et al., 1989b). The data were collected from crystals grown by the vapor diffusion method. In the vapor diffusion method, a 5-µL solution of 15 mg/mL protein in 50 mM Tris buffer, pH 7.5, was mixed with 5 µL of 50 mM Tris buffer, pH 7.5, containing 15% PEG 3500, 1 mM MgCl, and 0.5 mM EDTA, and suspended over 0.7 mL of the same buffer with 15% PEG 3500. Different additives, such as 20 mM Fru-6-*P* and/or 20 mM AMP were also

used. The crystals, which grew within 1–2 weeks to typical dimensions of 0.5 × 0.5 × 0.4, were harvested and used directly for data collection.

Data collection and processing

The diffraction data were collected with the Area Detector Systems (San Diego, California) Multiwire Area Detector MARK II system at the Crystallographic Facility in the Chemistry Department of Boston College. The two area detector system is controlled by a DEC ALPHA 3300 computer. The system is mounted on a Rigaku RU-200 rotating-anode X-ray generator operated at 50 kV and 150 mA. The detectors were positioned at 12 degrees and 32 degrees, with crystal to detector distances of 700 mm and 680 mm, respectively. This experimental setup allows the collection of the data to 2.0-Å resolution without repositioning the detectors. Two independent data sets were collected on T state crystals of the enzyme. Individual data collection lasted typically 38 h. Each data set was collected on a single crystal with overall decay of 19% and 16%, respectively. The data collection procedures resulted in 86% and 88% complete data sets to 2.2 Å and 2.05 Å resolution (no sigma cut off).

The crystals of the mutant in the R state did not diffract as well as the crystals for the T state, and initially scattered weakly to ~6 Å. After several days in the capillary, they stabilized and their diffracting power improved so the data could be collected to approximately 2.8-Å resolution.

The data were collected on one crystal and gave a data set 97% complete to 2.75-Å resolution. Because of the detector setup, reflections were collected to 2.2 Å, but usable data extended to about 2.75 Å. The diffraction data were merged using the software provided by Area Detector Systems (Hamlin et al., 1981). A scale factor was calculated for multiple measurements and symmetry-related reflections. Those measurements deviating substantially from the average for a particular reflection were deleted. This merging and editing procedure was repeated until the R_{merge} converged. The crystal and data collection statistics for all data sets are presented in Table 1.

Structural refinement

The initial models used to begin the structural refinement were the coordinates of the wild-type pig kidney Fru-1,6-*P*₂ase in the T and R forms, respectively (Brookhaven Protein Data Bank files: 1frp, 5fbp), with metals, ligands, and water molecules removed. All three crystal structures were refined using X-PLOR (Brünger, 1992; Molecular Simulations, Inc.) running on Silicon Graphics Indigo II workstations at Boston College. The initial *R*-factors were 0.36, 0.35, and 0.48 for the T state (2.2 Å), T state (2.05 Å), and of the R state (2.75 Å) structures, respectively. Subsequent positional refinement and temperature factor refinement improved the *R*-factor and stereochemistry. At this stage, the ligands Fru-6-*P* and AMP were built according to the calculated electron density ($2F_o - F_c$) and ($F_o - F_c$) maps using CHAIN (Sack, 1988). The structures were then further refined by cycles of positional refinement, temperature factor refinement, and simulated annealing using initial temperatures up to 1,000 K.

The solvent water molecules were then added automatically by IMPLOR (T. Tibbitts, unpubl.) and the final structure was obtained by manual modeling. During subsequent refinement,

the temperature factors of the waters were monitored carefully, and water molecules that had temperature factors above $90 \text{ \AA}^2$ were deleted. Additional solvent water molecules were added based upon difference Fourier maps ($F_o - F_c$) at several stages, and approximately 160 waters are present in each of the refined structures of the T state and approximately 80 for the R state (Table 2). Simulated annealing omit maps (Brünger, 1992) were also used to determine the positions of side chains in the active site region of these structures.

The quality of the models were monitored by the program PROCHECK (Laskowski et al., 1993). The molecular fragments 1–8 and 62–70, which usually have very weak electron density and were not refined previously (Ke et al., 1991b), were modeled and refined in our T state structures. The electron density for these fragments was weak, but in the structure reported here were refined to provide a better description for the model and its conformational limitations imposed by the existing structure. Additionally, residues Ala-336 and Lys-337 were added to the structure, in accord with amended primary sequence (Williams & Kantrowitz, 1992). The coordinates for the two T state and one R state structures of the Arg-243 → Ala Fru-1,6- P_2 ase have been deposited with Brookhaven Protein Data Bank as 1rdy, 1rdx, and 1rdz, respectively.

Evaluation of the refined structures and electrostatic calculations aimed at explaining the kinetic results

Final statistics and superimpositions to obtain RMSD were calculated in X-PLOR. Molecular surface calculations, electrostatic field calculations and visualization were carried out with the program GRASP (Nicholls, 1992). Initially the field was calculated from the starting model of the wild-type enzyme (1frp) and from the final structure of the refined T state form of the Arg-243 → Ala enzyme. The comparison of these fields showed that the omission of fragments 1–9, 62–69, and 336–337 influenced the shape and magnitude of the field at the active site. These fragments contain numerous charged residues such as Asp-2, Asp-7, Asp-64, Asp-68, Lys-337. Therefore, the final refined structure of the mutant was used for comparison without and with the Arg side chain modeled in a similar position to that observed in the native enzyme. The electrostatic field was calculated with program DELPHI (Gilson & Honig, 1987a, 1987b) as implemented in GRASP and visualized at the O1', O2', O6' phosphate groups. In the electrostatic calculations, the formal protein charges were used. The structure included one metal ion in both active sites, as refined in the native enzyme (PDB code 1frp). The total charge of the dimer with two metal ions was +8 and the mutant without Arg-243 was +6.

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