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A Direct Substrate–Substrate Interaction Found in the Kinase Domain of the Bifunctional Enzyme, 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase

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To understand the molecular basis of a phosphoryl transfer reaction catalyzed by the 6-phosphofructo-2-kinase domain of the hypoxia-inducible bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3), the crystal structures of PFKFB3•AMPPCP•fructose-6-phosphate and PFKFB3•ADP•phosphoenolpyruvate complexes were determined to 2.7 Å and 2.25 Å resolution, respectively. Kinetic studies on the wild-type and site-directed mutant proteins were carried out to confirm the structural observations. The experimentally varied liganding states in the active pocket cause no significant conformational changes. In the pseudo-substrate complex, a strong direct interaction between AMPPCP and fructose-6-phosphate (Fru-6-P) is found. By virtue of this direct substrate–substrate interaction, Fru-6-P is aligned with AMPPCP in an orientation and proximity most suitable for a direct transfer of the γ -phosphate moiety to 2-OH of Fru-6-P. The three key atoms involved in the phosphoryl transfer, the β,γ -phosphate bridge oxygen atom, the γ -phosphorus atom, and the 2-OH group are positioned in a single line, suggesting a direct phosphoryl transfer without formation of a phosphoenzyme intermediate. In addition, the distance between 2-OH and γ -phosphorus allows the γ -phosphate oxygen atoms to serve as a general base catalyst to induce an “associative” phosphoryl transfer mechanism. The site-directed mutant study and inhibition kinetics suggest that this reaction will be catalyzed most efficiently by the protein when the substrates bind to the active pocket in an ordered manner in which ATP binds first.

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Introduction

Every mammalian cell is equipped with one of the four distinct gene-encoded tissue isoforms of

the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB): liver, PFKFB1; heart, PFKFB2; testis, PFKFB4; and inducible forms, PFKFB3.^{1–4} The PFKFB tissue isoforms optimize glucose utilization in a tissue-specific manner. Although these isoforms share a high level of sequence identity in the core catalytic domains (>85%), each isoform has different kinetic properties and responds differently to upstream regulatory signals. This molecular differentiation results from the highly divergent N and C-terminal regulatory domains and the sequence differences in the second shell residues surrounding the active site pockets of the catalytic domains.^{5–7} Unlike the other three isoforms, PFKFB3 is expressed ubiquitously upon the onset of hypoxic stress and found to be identical

Abbreviations used: PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; 2-Kase, 6-phosphofructo-2-kinase; 2-Pase, fructose-2,6-bisphosphatase; Fru-6-P, fructose-6-phosphate; Fru-2,6-P₂, fructose-2,6-bisphosphate; PEP, phosphoenolpyruvate; AMPPCP, β,γ -methylene-adenosine 5'-triphosphate; GntK, gluconate kinase.

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with the forms isolated from placenta, pancreatic beta-cell, and brain.^{8–12}

PFKFB controls the cellular concentration of fructose-2,6-bisphosphate (Fru-2,6-P₂) by modulating the two mutually opposing catalytic activities of Fru-2,6-P₂ synthesis (2-Kase, 6-phosphofructo-2-kinase) and hydrolysis (2-Pase, fructose-2,6-bisphosphatase), which reside in a single protein molecule as two separate domains.^{1,2,13} Fru-2,6-P₂ is the most potent allosteric activator of phosphofructokinase, the rate-limiting enzyme of glycolysis, and the inhibitor of fructose-1,6-bisphosphatase, the rate-limiting enzyme of gluconeogenesis.^{1,2,13} As such, changes in the Fru-2,6-P₂ concentrations by PFKFB cause changes in the glycolytic rates in all tissues, and both glycolysis and gluconeogenesis in liver. Ultimately, PFKFB controls cellular glycolysis and gluconeogenesis to optimize glucose utilization in a tissue-specific manner. To carry out this function, the two catalytic activities in PFKFB are exquisitely regulated by various metabolic products and signal transduction-dependent phosphorylation, such that the resulting predominant activity determines the final concentration of Fru-2,6-P₂ and, ultimately, the rates of glycolysis in all tissues, and both glycolysis and gluconeogenesis in the liver.^{1,2,13}

Due to its biological significance and the complexity of regulation of PFKFB isoforms, and their potential applicability to medicine, the PFKFB enzyme system has long been a focus of structure/function studies. As a result, the structures of PFKFB1 and PFKFB4 have become available and, thus, an understanding of a molecular differentiation of the PFKFB isoforms has been reached.^{6,7} Structure/function studies using the separate 2-Pase domain allowed understanding of the reaction mechanism of 2-Pase at the molecular level.^{14,15} However, despite long, continuous efforts, understanding of the 2-Kase reaction at a molecular level has been lacking. Although significant progress was made using PFKFB4, no detailed information on catalytic mechanism has been available to date except for the locations of substrate-binding sites and a few catalytic site residues.^{7,16} This slow progress is largely due to the insufficient molecular level information on the interactions made by Fru-6-P in the 2-Kase active pocket. No attempt to achieve 2-Kase•Fru-6-P complex has been successful to date. We have studied the PFKFB enzyme system and made significant contributions to understanding the structure/function relationships of this enzyme system at the molecular level. As part of our continued efforts to understand the differences between the PFKFB isoforms at the molecular level, we recently determined the crystal structures of the human tumor/inducible form of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) in a near native state (PFKFB3•ADP•EDTA, PDB accession code 2AXN) and its product complex of PFKFB3•ADP•Fru-2,6-P₂ (PDB accession code 2DWP).⁵

It has been suggested that PFKFB isoforms are involved in metabolic disorders, including diabetes, obesity, and cancer. High levels of blood glucose are

related to low concentrations of hepatic Fru-2,6-P₂ and can be ameliorated by activation of 2-Kase activity of PFKFB1. The high 2-Kase activity of PFKFB3 is considered as a driving force of cancer glycolysis. A small molecule that can inhibit PFKFB3 2-Kase may serve as a new cancer drug. Thus, understanding of 2-Kase catalytic mechanism at the molecular level is urgent for drug development. To study the unknown molecular catalytic mechanism of 2-Kase, we determined the structure of the PFKFB•Fru-6-P complex in the presence of the non-reactive ATP-analogue AMPPCP (β,γ -methyleneadenosine 5'-triphosphate), employing the PFKFB3 isoform, and that of an inhibitory complex of PFKFB3•ADP•PEP (phosphoenolpyruvate). These structures were compared with that of the previously attained PFKFB3•ADP•Fru-2,6-P₂ complex. Although the structure of this product complex was introduced originally to confirm the location of the active pocket,⁵ its functional details have not been reported. Thus, the functionality of this product complex is discussed here, together with new studies on the catalytic mechanism of the kinase reaction. The results of structure/function studies are discussed here.

Results and Discussions

Overall structure

As shown in the ribbon diagram of a single subunit of PFKFB3 complexed with ADP and PEP, PFKFB3 has two separate catalytic domains (Figure 1(a)). As in the PFKFB3•ADP•EDTA complex, the N-terminal residues 4–15 of both complexes form a β -hairpin structure to make contacts with the C-terminal 2-Pase domain at the area where the residues involved in binding the 6-phosphate moieties of both substrate and product are collectively located. In this way, the self-regulatory effect by the N terminus on the 2-Pase activity is not affected by the N-terminal 2-Kase liganding.⁵ Even Fru-6-P binding to the 2-Kase active pocket does not involve any conformational change in the N terminus, in contrast to a previous suggestion based on a fluorescence study.¹⁷ The structures of the C terminus (residues 446–520) remain disordered,⁵ despite the varied liganding states of the 2-Kase.

Possible conformational changes in the 2-Kase core domain by the experimentally varied liganding conditions were investigated by superposition of the structures. The structures of the PFKFB3•AMPPCP•Fru-6-P complex and the PFKFB3•ADP•PEP complex were superposed onto that of the PFKFB3•ADP•Fru-2,6-P₂, using least-squares error transformations of the C α positions. The resulting superposed structures of the 2-Kase domains are shown in Figure 1(b). No residue diverged by more than 0.5 Å, except for those forming the ATP loop (residues 168–179) and the F-6-P loop (residues 72–84). Relatively large differences in the ATP and Fru-6-P loops seem to be caused by variations in molecular sizes of the ligands bound

Figure 1. Overall folding of PFKFB3 and ligand binding. (a) A ribbon diagram of monomeric PFKFB3 is shown to represent the overall folding of the protein. ADP is bound to the 2-Kase (red) active pocket and Fru-6-P to the 2-Pase (blue). The N-terminal regulatory domain is shown in green. Unless specifically mentioned, all structure Figures were made using MolScript v2.1.2,⁴⁵ and rendered using Raster3D v2.7.⁴⁶ (b) A comparison of 2-Kase fold in different liganding states. The folding patterns are represented by a tracing of C α atoms (residues 34–246): PFKFB3•AMPPCP•Fru-6-P in green, PFKFB3•ADP•Fru-2,6-P₂ in blue, and PFKFB3•ADP•PEP in magenta. The ATP site and the Fru-6-P site are represented by the ball-and-stick models of ADP and Fru-2,6-P₂.

Figure 2. Binding of AMPPCP and Fru-6-P to the 2-Kase catalytic pocket. (a) A stereo view of the $|F_o| - |F_c|$ omit electron density map from the PFKFB3•AMPPCP•Fru-6-P complex. The map is calculated in the absence of ligands and contoured around the γ -phosphate moiety of AMPPCP and Fru-6-P at 2.5σ levels. The incorporated ligand structure is from the final model. The electron density maps are drawn using BobScript v1.4b. (b) Coordination of Mg²⁺ is shown and the relative position of Fru-6-P is provided. (c) A stereo view of the binding of Fru-6-P to the 2-Kase catalytic pocket. For convenient comparison, Fru-2,6-P₂ and the interacting residues are drawn in light grey after superposition of the structures of PFKFB3•AMPPCP•Fru-6-P and PFKFB3•ADP•Fru-2,6-P₂ complexes. (d) The direct interactions between AMPPCP and Fru-6-P in the 2-Kase active site pocket. The dotted lines represent hydrogen bonds or salt-bridges. The interactions between the γ -phosphate moiety of AMPPCP and the protein are shown. ATP bound to PFKFB1 in the absence of Mg²⁺ is superposed to show the importance of Mg²⁺. A grey dotted line is drawn between the bridge oxygen atom in ATP and O2 of Fru-6-P to emphasize the in-line positioning of the two oxygen and the γ -phosphorus atoms.

Figure 2 (legend on previous page)

to the Fru-6-P site. However, the extents are not comparable to those of substrate-induced conformational changes observed in PFKFB4 (the testis isoform),¹⁶ or in other similarly folded small molecule phosphoryl transferases.^{18–21} The shikimate, the gluconate, and the nucleoside monophosphate kinases undergo a conformational shift between the “closed” and “open” states, depending on binding of substrates or products. In PFKFB4, the last turn of the $\alpha 5$ switch helix (residues 158–171) of the ATP loop undergoes unwinding upon displacing ADP with ATP. This unwinding allows a key catalytic residue, Lys168, to interact with the bridge oxygen atom of β and γ -phosphate groups of ATP, otherwise pointing to the surface.¹⁶ A conformational change similar to

PFKFB4 has been observed from the *c-ras* protein.²⁰ This switch motion has been hypothesized to induce a conformation necessary for the subsequent binding of Fru-6-P, a mechanism for ordered binding of the two substrates in the case of PFKFB4. However, this helix is always unwound, regardless of ADP or ATP binding, and the critical catalytic interaction made by Lys168 is always present in PFKFB1 and in PFKFB3, due to their sequence-based structural differences from PFKFB4.^{5,6}

Binding of substrates and products

In previous trials to achieve a Fru-6-P complex using ATP γ S as an ATP analogue, the reaction

proceeded and resulted in the PFKFB3•ADP•Fru-2,6-P₂ product complex.⁵ To abolish this unwanted reaction, non-reactive β,γ -methylene-adenosine 5'-triphosphate (AMPPCP) was used in the current study. A resulting $|F_o| - |F_c|$ omit map generated from the PFKFB3•AMPPCP•Fru-6-P complex in the absence of ligands revealed electron densities representing AMPPCP, a Mg²⁺, and Fru-6-P (Figure 2(a)). The position of Mg²⁺ was confirmed by its coordination in addition to the electron density (Figure 2(b)). In this structure, the Mg²⁺ is coordinated by two phosphate oxygen atoms from AMPPCP, three solvent molecules, and OG1 of Thr48 with distances in a range of 2.2–3.0 Å, forming an octahedral coordination.

As compared in Figure 2(c), Fru-6-P occupies the same binding site as Fru-2,6-P₂ in the PFKFB3•ADP•Fru-2,6-P₂ complex, which is embedded in this Figure.⁵ The substrate (Fru-6-P) and the product (Fru-2,6-P₂) share a number of residues for binding. These interactions are summarized in Table 2. This positional overlap suggests that Fru-6-P in the PFKFB3•AMPPCP•Fru-6-P complex binds to the catalytic site, and that this complex indeed mimics the reactive ternary substrate complex. Fru-6-P binds to the active pocket through its 6-phosphate moiety, which interacts with Arg98, Thr126, Arg132, and Tyr193 as Fru-2,6-P₂ does, but in a manner slightly different from that of Fru-2,6-P₂. Tyr193 is omitted from the Figure to enhance visual clarity. The interactions made by Arg74 with O3 and O4, and by Arg189 with O1, and by Tyr193 with O5 appear critically important for a phosphoryl transfer catalyzed by this enzyme. By virtue of these hydrogen bonds, O1 and O2 of the furanosyl ring of Fru-6-P are oriented by the γ -phosphate group of ATP (Figure 2(d)). Moreover, the interaction between O1 and Arg189 serves to select only β -anomeric Fru-6-P as substrate, as demonstrated in an NMR study of PFKFB1.²²

Similar to Fru-6-P, major interactions of Fru-2,6-P₂ with the protein occur through its 6-phosphate moiety. A significant difference from Fru-6-P is that only O3 interacts with Arg74 and O4 with Arg98 instead of Arg74. This difference is caused by the added phosphate at the O2 position as the result of the phosphoryl transfer reaction catalyzed by this protein. The 2-phosphate does not have any significant interaction with the protein, except for with Lys168. But, as will be described later, Lys168 remains in the same position regardless of the liganding states of the protein. Because of the negative charges in the β -phosphate moiety of ADP and the 2-phosphate of Fru-2,6-P₂, the two products repel each other. To minimize this repulsive force, it appears that the 2-phosphate rotates downward to the entrance to the catalytic pocket after completion of the phosphoryl transfer. There, 2-phosphate has a total of four hydrogen bonds with the neighboring solvent molecules (data not shown) that are located at near the entrance to the catalytic pocket. This implies that 2-phosphate has more chances to interact with water molecules from the bulk solvent. Even-

tually, it will lead to solvation of 2-phosphate and may help the release of Fru-2,6-P₂ from the active pocket upon completion of a catalytic cycle.

Surprisingly, no additional amino acid residue is employed for the interactions with the γ -phosphate moiety of AMPPCP (Figure 2(d)). Although Lys168 and Lys47 are positioned at distances of 2.9 Å from the γ -phosphate to provide salt-bridges, the conformations of Lys168 and Lys47, which also provide salt-bridges to the β -phosphate in other ADP complexes, are not changed significantly from those in other ADP complexes. It appears that the γ -phosphate oxygen atoms are simply inserted into the bonding field of the two positively charged residues. Lys47 is a member of the Walker A motif, consisting of ⁻⁴¹GLPARGKT⁴⁸-, which is shared in all PFKFB isoforms.⁷ The previous functional study showed that site-directed mutagenesis of Lys47 causes a decrease in k_{cat} by an order of magnitude, suggesting its critical role in catalysis.²³ Its dual interactions with both the β and γ -phosphate suggest that Lys47 may be involved in stabilization of the transition state. Functional characterization of Lys168 was done previously,²⁴ and its critical role in catalysis is confirmed here. The detailed description of this study is given in the following section. Notably, the position of Lys168 is not affected even when the residue is exposed to the β and γ -phosphate-bridging carbon atom in AMPPCP instead of an oxygen atom in ADP or ATP. This is probably because of the added interaction with the γ -phosphate oxygen atoms. The unvaried conformation of Lys168 may be facilitated by its hydrophobic intercalation between Val167 and Phe186 (data not shown).

Co-liganded with AMPPCP, Mg²⁺ provides two salt-bridges to the two oxygen atoms, O2B and O2G, from the β and γ -phosphate moieties of AMPPCP with distances of 2.6 Å and 2.4 Å, respectively, as pointed out earlier (Figure 2(b)). Providing interactions with the phosphate oxygen atoms, Mg²⁺ also seems to have an important role in the 2-Kase-catalyzed phosphoryl transfer. As shown in Figure 2(d), Mg²⁺ makes a major contribution to orientation of the γ -phosphate suitable for an in-line phosphoryl transfer. The γ -phosphate group of ATP complexed with PFKFB1 in the absence of Mg²⁺ (PDB accession code 1K6M),²³ which is embedded, is located in a position different from that in the presence of Mg²⁺, explaining why this protein family requires Mg²⁺ for its catalytic reaction. The γ -phosphate group of ATP γ S complexed with PFKFB4 in the presence of Mg²⁺ (PDB accession code 1BIF)²³ shares the same position as that in the current ternary PFKFB3•AMPPCP•Fru-6-P complex, supporting the function of Mg²⁺ suggested here.

Direct substrate–substrate interaction

As described above, the γ -phosphate group of AMPPCP has only modest interactions with the protein. However, it was found that this phosphate moiety mediates a strong direct substrate–substrate

Table 1. Statistics of reflection data and structure refinements

Liganding	PFKFB3•AMPPCP• Fru-6-P	PFKFB3•ADP• PEP
Space group	<i>P</i> 6 ₅ 22	<i>P</i> 6 ₅ 22
Unit cell dimensions		
<i>a</i> = <i>b</i> (Å)	101.99	102.29
<i>c</i> (Å)	258.07	258.62
Resolution range (Å)	30.0–2.7	30.0–2.25
No. reflections used	21,941	38,937
Completeness (%)	96.4 (64.8)	99.7 (99.1)
Redundancy	9.8 (4.0)	7.0 (4.2)
<i>I</i> / σ (<i>I</i>)	16.2 (1.6)	26.2 (3.7)
<i>R</i> _{sym}	0.135 (0.473)	0.054 (0.334)
<i>R</i> _{crys}	0.224	0.214
<i>R</i> _{free}	0.264	0.243
No. amino acids	433	449
No. protein atoms	3520	3657
No. hetero atoms	64	52
No. water molecules	296	298
r.m.s.d. from ideal		
Bond lengths (Å)	0.006	0.009
Bond angles (deg.)	1.2	1.4
Dihedral angles (deg.)	21.9	22.1
Mean Bfactor		
Protein atoms (Å ²)	56.5	40.0
Hetero atoms (Å ²)	53.1	37.9
Water atoms (Å ²)	53.1	51.2

$R_{\text{sym}} = \sum_h (\sum_j |I_{h,j} - \langle I_h \rangle| / \sum_j I_{h,j})$, where *h*=set of Miller indices, *j*=set of observations of reflection *h*, and $\langle I_h \rangle$ =the mean intensity. $R_{\text{crys}} = \sum_h ||F_{o,h}| - |F_{c,h}|| / \sum_h |F_{o,h}|$. R_{free} was calculated using 10% of the complete data set excluded from refinement. The numbers in parentheses represent values from the highest resolution shell (2.75–2.70 Å).

interaction between AMPPCP and Fru-6-P in the PFKFB3•AMPPCP•Fru-6-P structure, as shown in Figure 2(d). In this complex, all three γ -phosphate oxygen atoms make direct interactions with O1 and O2 of Fru-6-P. The O3G and O2G form hydrogen bonds with O2 of Fru-6-P, the phosphoryl acceptor, with distances of 2.8 Å and 2.9 Å, respectively. And O1G interacts with O1 at a distance of 2.7 Å. As a consequence of this strong direct interaction, the distance between AMPPCP and Fru-6-P in the pseudo-substrate complex is 0.4 Å shorter than that between ADP and Fru-6-P₂ in the product complex. This interligand interaction may also have caused a 21°-rotated position of the furanose ring of Fru-6-P and a different position of O1 of Fru-6-P relative to Fru-2,6-P₂. The resulting torsional strain may have caused differences between Fru-6-P and Fru-2,6-P₂ in their interactions through the 6-phosphate moieties (Figure 2(c)).

The direct interaction between AMPPCP and Fru-6-P provides an interesting insight into the phosphoryl transfer mechanism catalyzed by 6-phosphofructo-2-kinase of the PFKFB protein family. In addition to the hydrogen bonds made between the furanosyl oxygen atoms and the protein, a direct intersubstrate interaction between O1 and O2 and the γ -phosphate causes O2 of Fru-6-P to locate nearest to the γ -phosphorus atom. In the current PFKFB3•AMPPCP•Fru-6-P structure, the phosphoryl acceptor, O2 of Fru-6-P, is located only 3.1 Å away from the γ -phosphorus of AMPPCP.

O2 is the most closely located non-covalent atom from γ -phosphorus and, as a result, can serve as the most efficient acceptor of the γ -phosphate from ATP. Furthermore, O2, the γ -phosphorus, and the β and γ -phosphate bridge oxygen atoms are located on a near a single line with a measured angle of 164°, as indicated by a grey dotted line in Figure 2(d). The distance between the phosphoryl acceptor and the γ -phosphorus, and the geometric arrangement of the three atoms together suggest an “in-line” transfer of phosphate accompanied with inversion of phosphorus configuration.^{25,26} This structural observation coincides with the previous biophysical observation on the stereochemical course of phosphoryl transfer catalyzed by PFKFB1.²² When the configuration of transferred phosphorus atom was tested by ³¹P NMR spectroscopy, using [γ -(S)-¹⁶O, ¹⁷O, ¹⁸O]ATP as probing substrate, an in-line transfer with a net inversion of configuration was observed. From this observation, a double displacement ping-pong mechanism was suggested, and formation of a covalent phosphoenzyme intermediate was ruled out. A similar conclusion suggesting an in-line sequential mechanism was made from an isotope exchange reaction study performed by a different group.²⁷ The three independent studies together suggest that PFKFB 2-Kase reaction follows an in-line phosphoryl transfer.

In the current structure, the distance between the phosphoryl donor, the bridge oxygen atom, and the acceptor, O2 of Fru-6-P, is 4.8 Å. If the estimated coordinate error of 0.42 Å is added, this distance is still within 5.22 Å. This geometry suggests that the in-line transfer is likely to follow an associative mechanism^{26–29} in which O2 of Fru-6-P functions as the attacking nucleophile. For O2 to function as a nucleophile, a base catalyst that deprotonates –OH to form an active nucleophile, has to be located within hydrogen bond distance. From a search for

Table 2. Interactions of the Fru-6-P site-bound ligands with the protein and ATP

Protein atoms	Fru-6-P	Fru-2,6-P ₂	PEP	EDTA
Lys47	NZ			O5 (2.8)
Arg74	NE	O3 (2.8)	O2 (2.6)	O4 (2.7)
	NH2	O4 (3.1)	O2 (3.0)	O2 (2.6)
Arg75	NH1	O3 (2.7)		O7 (3.1)
	NH2			O8 (3.0)
Arg98	NE		O3P (2.7)	
	NH1		O2P (3.2)	
	NH2	O4P (3.1)		O4 (3.2)
Thr126	N	O6 (2.4)	O6 (3.1)	O1P (3.5)
	OG1	O6P (2.1)	O6P (2.6)	O2P (2.8)
Arg132	NH1	O5P (3.0)	O5P (2.5)	O3 (3.2)
Lys168	NZ		O1P (3.2)	
Arg189	NE	O1 (3.4)	O1 (3.2)	
	NH2		O1 (2.9)	O1 (3.8)
Tyr193	OH	O6 (2.7)	O4P (2.6)	O2P (2.6)
		O5 (3.5)	O5 (3.5)	
*ATP	O1G	O1 (2.7)		
	O2G	O2 (2.9)		
	O3G	O2 (2.8)		

*ATP: atoms from the PFKFB3+bound AMPPCP. Numbers in parentheses are distances (in Å).

such a base catalyst, a water molecule that is hydrogen-bonded to Asn69 and involved in coordination of Mg^{2+} was found 3.1 Å away from O2 (Figure 2(c)). This water molecule may be able to serve as a base catalyst if activated by a Lewis acid function of Mg^{2+} ³⁰ and/or by Asn69. Divalent cations other than Mg^{2+} usually slow the reaction but do not abolish reactivity.³⁰ A previous site-directed mutagenesis study suggested that Asn69 does not have a significant role in catalysis.³¹ Both observations diminish the functional value of this water molecule as a base catalyst. On the other hand, as described above, the γ -phosphate of AMPPCP with multiple negative charges forms a direct and stronger interaction to O2. The electron-rich γ -phosphate that is located within 3 Å may have a stronger potential than Mg^{2+} -coordinated water in deprotonating 2-OH of Fru-6-P, or in functioning as a base catalyst. Moreover, such substrate-assisted mechanism has been observed from the studies of Rab11 (a small GTP-binding protein) and GntK (gluconate kinase).^{19,32} Since PFKFB 2-Kase folding is very similar to these proteins with the folding similarity Z score of 13.8. from the DALI server† calculation, its catalytic mechanism is likely similar to theirs. Taken together, we suggest that the nucleophile formation in PFKFB 2-Kase is performed by a substrate-assisted mechanism.

Interaction of the β,γ -phosphate bridge oxygen atom with protein

As pointed out above, Lys168 is positioned to interact with the β,γ -phosphate bridge oxygen (carbon in AMPPCP) atom, and the main-chain N of Ala44 is located approximately 3.0 Å away from the bridge oxygen atom, the phosphoryl donor (Figure 2(d)). Their locations suggest that they have an important role in the catalytic mechanism. To test this possibility, we engineered and kinetically characterized a total of five site-directed mutant proteins: Lys168→A, Lys168→N, and Lys168→R and Ala44→G and Ala44→V. Because the main-chain N of Ala44 interacts with the bridge oxygen, it is reasonable to consider that varying the size of the side chain of this residue is accompanied by varied strengths/distances of the interaction between N and the bridging oxygen. Since the side-chain of this residue makes hydrophobic interactions with a local hydrophobic pocket formed by Val160, Ile164, and Ile190 (data not shown), addition of a small hydrophobic side-chain does not cause any significant folding problem. Circular dichroism spectroscopy was used to test all mutants for proper folding. As summarized in Table 3, changes in this distance introduced by mutagenesis cause significant changes only in K_m for substrates. The accompanying changes in k_{cat} are insignificant and less than 20%. The K_m values for ATP and Fru-6-P of Ala44→G are decreased by approximately 20-fold

Table 3. Kinetic properties of site-directed mutants

Mutant	k_{cat} (s^{-1})	K_m^{ATP} (μM)	K_m^{F-6-P} (μM)
Wild-type	0.14 ± 0.0034	16.9 ± 1.7	10.2 ± 2.2
Ala44→G	0.19 ± 0.0027	0.87 ± 0.09	3.8 ± 0.62
Ala44→V	0.17 ± 0.0057	140.2 ± 2.1	204 ± 22.2
Lys168→R	< 0.0003	N.D.	N.D.
Lys168→A or N	N.D.	N.D.	N.D.

N.D. not determinable.

and threefold, respectively, and those of Ala44→V are increased by eightfold and 20-fold, respectively. As such, kinetic observation of the Ala44 mutants suggests that the main-chain N of Ala44 is involved mainly in ATP binding rather than catalytic turnover. However, it is notable that changes in K_m for ATP are accompanied by changes in K_m for Fru-6-P, supporting the direct ATP/Fru-6-P interaction.

On the other hand, mutagenesis of Lys168 caused a complete abolition of the catalytic activity, except for Lys168→R, which retains only 0.2% of that of the wild type, confirming previous observations.²⁴ It is evident that Lys168 has a crucial role in the phosphoryl transfer reaction. This rationale suggests a similarity to a function of the “arginine finger” provided by GAP (GTPase-activating protein) to the GTPase activity of *ras*-like small G_{α} proteins in the *ras*•GAP complex.^{20,33,34} This arginine provides a salt-bridge to the bridge oxygen atom of GTP bound to the *ras* protein, which otherwise does not exist. The arginine residue inserted into the *ras* active pocket functions as the transition state stabilizer to cause an increase in the GTPase activity in an order of magnitude.³³ Similar observations on the positively charged residues interacting with the bridge oxygen atom have been made from the shikimate kinase and the gluconate kinase.^{19,35} The positional similarity between Lys168 and these residues suggests that Lys168 also functions as the transition state stabilizer. Supporting this rationale, Lys168 also interacts with the γ -phosphate oxygen atom, with a distance of 2.4 Å. This observation suggests that Lys168, together with Lys47, is involved in stabilization of the pentavalent phosphorane, which is transiently formed immediately after the nucleophilic attack of O2 of Fru-6-P on the γ -phosphorus of ATP. Consequently, mutagenesis of Lys168 results in a severely compromised catalytic mechanism and may explain the complete loss of 2-Kase activities of the Lys168 mutant proteins.

Suggested catalytic mechanism

On the basis of the structure/function studies, a possible molecular catalytic mechanism is summarized as follows. A nucleophile is produced by deprotonation of 2-OH of Fru-6-P by the negatively charged γ -phosphate oxygen atoms. Its nucleophilic attack on the γ -phosphorus initiates the reaction and is followed by formation of a transient high-energy pentavalent phosphorane. For the reaction to proceed, negative charges developed on the three equatorial oxygen atoms of the

† <http://www.ebi.ac.uk/dali/fssp/>

phosphorane must be stabilized by Lys168, Lys47, and Mg^{2+} . The proton extracted from 2-OH by the γ -phosphate may be involved in stabilization of this intermediate. Finally, the diester bond between the bridge oxygen and the γ -phosphorus is broken by redistribution of the electrons of the equatorial oxygen atoms. A possible back attack by the leaving group, ADP, is prevented by concomitant protonation of the bridge oxygen. This mechanism is summarized in Figure 3. This mechanism is quite similar to those adopted by a number of small-molecule phosphoryl transferases, including gluconate kinase (GntK),¹⁹ and the *ras*-like small GTP-binding protein Rab11.³² Rab11 and GntK share a "substrate-assisted" mechanism in which the nucleophile is formed as the result of direct substrate-substrate interactions. As in PFKFB, Mg^{2+} has a role in aligning the γ -phosphate in-line with respect to the phosphoryl acceptor in GntK.

PEP inhibition and substrate-binding order

Phosphoenolpyruvate (PEP) has been suggested as an allosteric inhibitor of PFKFB3.³⁶ Phosphoryla-

tion of Ser460 by protein kinase A or AMP-dependent protein kinase causes a conformational change such that the PEP allosteric inhibition is alleviated to increase the activity. To find this intriguing allosteric site and to understand the molecular mechanism of regulatory phosphorylation of PFKFB3, we determined the structure of PFKFB3•PEP complex. As shown in a $|F_o| - |F_c|$ omit map, PEP binding was very obvious (Figure 4(a)) but surprisingly located at the Fru-6-P site in the active pocket (Figure 4(b)). There, the phosphate moiety of PEP is almost completely overlapping with that of the 6-phosphate moiety of Fru-6-P or Fru-2,6-P₂. The unexpected positioning of PEP suggests strongly that PEP functions as a competitive inhibitor against Fru-6-P, rather than as an allosteric inhibitor. This positioning is consistent with the observation from a very early functional study on PFKFB1 that suggested PEP serves as a competitive inhibitor.³⁷ Inhibition kinetic studies of PEP were carried out to confirm this structural observation. As clearly shown in a double-reciprocal plot (Figure 5), PEP behaves like a competitive inhibitor against Fru-6-P with apparent K_{is} of 8.3 (± 0.9)

Figure 3. A cartoon of the suggested catalytic pathway. In the clockwise direction, 2-OH of Fru-6-P is deprotonated by the γ -phosphate moiety of ATP and a nucleophilic attack occurs. An intermediate pentavalent phosphorane is formed, and the negative charges generated on the planary oxygen atoms are stabilized by Lys47, Lys168, and Mg^{2+} , and maybe a H^+ extracted from 2-OH. Redistribution of the phosphorane electrons breaks a diester bond between the bridge oxygen and γ -phosphorus atoms. The leaving group is stabilized by a H^+ . Eventually, the leaving group (ADP) is stabilized through a salt-bridge to Lys168 to finish the reaction.

Figure 4. PEP binding to PFKFB3. (a) The $|F_o| - |F_c|$ omit electron density map. The map is calculated in the absence of ligands and contoured at 2.5σ . (b) A stereo view of the interactions of PEP with the 2-Kase active site pocket is shown. The dotted lines represent hydrogen bonds or salt-bridges. To show its position in the 2-Kase active pocket, the structure of PFKFB3•AMPPCP•Fru-6-P complex is superposed and Fru-6-P (light gray) is shown.

μM (Table 4). Thus, the structure and function studies indicated unequivocally that PEP is a competitive inhibitor.

As described earlier, mutagenesis of Ala44 showed that Fru-6-P binding is sensitive to that of ATP, a clue to suggest “ordered” binding of substrates. This contradicts our previous speculation that substrates bind to PFKFB3 in a random manner. This was based on the absence of a switch motion and that Arg181, that causes structural coupling of

the two substrate loops in PFKFB4, is Cys in PFKFB3.⁵ To investigate this ambiguity, inhibition kinetics was carried out using a product (ADP) and a substrate analogue (PEP) as inhibitors. Since using the other product, Fru-2,6-P₂, as an inhibitor is not suitable in the current 2-Kase assay system, PEP, which is redefined as a competitive inhibitor of Fru-6-P as described above, was adopted as a Fru-6-P analogue in the inhibition kinetics. As shown in the double-reciprocal plots in Figure 5 (and see Table 4),

Figure 5. 2-Kase inhibition by ADP and PEP. Double-reciprocal plots were generated in which numbers by the lines represent inhibitor concentration (μM). (a) Inhibition by PEP with ATP as the variable substrate; (b) inhibition by PEP with Fru-6-P; (c) inhibition by ADP with ATP; (d) inhibition by ADP with Fru-6-P. Data were fit to one of three equations in Materials and Methods. The lines represent the calculated lines after fitting the data to the appropriate equation.

Table 4. ADP and PEP inhibition properties

Inhibitor	Variable substrate	Fixed substrate	K_{is} (μ M)	K_{ii} (μ M)	Inhibition
PEP	ATP	Fru-6-P		89.5 $\pm$ 9.8	UC
	Fru-6-P	ATP	8.3 $\pm$ 0.9		C
ADP	ATP	Fru-6-P	17.7 $\pm$ 2.5		C
	Fru-6-P	ATP	26.1 $\pm$ 8.2	127 $\pm$ 23	NC

Inhibition: UC, uncompetitive; C, competitive; NC, non-competitive.

a reaction product, ADP behaves as a competitive inhibitor against ATP (K_{is} = 17.7($\pm$ 2.5) μ M) and a non-competitive inhibitor against Fru-6-P with apparent K_{is} and K_{ii} of 26.1($\pm$ 8.2) μ M and 127($\pm$ 23) μ M, respectively. Meanwhile, PEP functions as an uncompetitive inhibitor against ATP with an apparent K_{ii} of 89.5($\pm$ 9.8) μ M in addition to its competitive inhibition against Fru-6-P, which is described above. Properties of the current inhibition kinetics suggest ordered binding of the two substrates with ATP first as in PFKFB4.¹⁶

Unlike PFKFB4 that undergoes the switch helix unwinding upon ATP binding, the current PFKFB3 structures suggest no significant ligand-dependent conformational changes that can be attributable to the structural basis of ordered binding. Thus, an explanation for the ordered substrate binding to PFKFB3 can be made by either of the following or a combination of both: A change in the protein conformation associated with binding of substrates may be too transient to be captured by the traditional crystallographic method we use. Or, the order may be related to the potent substrate-substrate interaction between O1 and O2 of Fru-6-P and the oxygen atoms of the γ -phosphate group of ATP. Assuming that the two substrates share a common entry route to the active site pocket, there is a possibility that a random binding of one substrate will cause a severe steric hindrance to binding of the other substrate. From this viewpoint, PFKFB1, which was previously assumed to follow random binding from a simple structural observation, needs further functional characterization.

Conclusion

To study the molecular basis of the phosphoryl transfer reaction catalyzed by the 6-phosphofructo-2-kinase domain of the bifunctional enzyme PFKFB1-4, the first crystal structure of a PFKFB isoform in a ternary substrate complex with Fru-6-P and ATP-analogue was determined, and a set of kinetic studies was carried out on the wild-type and several site-directed mutant proteins. To elucidate the molecular basis of the catalytic mechanism, the structure was compared with that of the protein in ternary product complex with Fru-2,6-P₂ and ADP. A direct substrate-substrate interaction through the γ -phosphate moiety of ATP and O1 and O2 of Fru-6-P, and the resulting geometry suggests an in-line phosphoryl transfer initiated by a substrate-assisted mechanism. It suggests also

that the γ -phosphate of moiety ATP is likely to be transferred to O2 of Fru-6-P in an associative manner, with Lys168 playing a critical functional role. With no significant change in protein conformation, the reaction still follows a sequential ordered binding of substrates with ATP first. The observations made from this study are mostly in agreement with a number of previous functional studies that were performed on different PFKFB isoforms. This, together with the fact that constellations of the catalytic site residues in the active pocket are conserved among all PFKFB isoforms, suggests that the molecular catalytic mechanism derived from this study is applicable to all other PFKFB isoforms. This study has provided a molecular basis of a physiologically important phosphoryl transfer reaction. Accordingly, this study may contribute to future development of PFKFB1 2-Kase activators and PFKFB3 2-Kase inhibitors, which may serve as potential therapeutics for diabetes and cancer, respectively. This study also enhances our understanding of differences in the structure/function relationships between the PFKFB isoforms at the molecular level.

Materials and Methods

2-Kase assay and kinetic analysis

To determine steady-state initial reaction rates, the 2-Kase reactions were performed first and the F-2,6-P₂ produced was measured according to the conventional method.³⁸ The 2-Kase reaction mixture (final volume of 0.2 ml) contained 20 mM Tris-HCl (pH 8.5), 3 mM DTT, 0.5 mM MgCl₂, and the indicated amounts of ATP, ADP, PEP, and Fru-6-P. The reaction was started by adding the mutant or wild-type enzyme and incubation at 25 °C for 20 min, and the concentration of Fru-2,6-P₂ was determined every 5 min. Fru-2,6-P₂ was measured spectrophotometrically at 340 nm using the potato pyrophosphate-dependent 6-phosphofructokinase (PP_i-PF1K) activation assay within 1 h of stopping the reaction by the addition of 125 mM NaOH. The PP_i-PF1K reaction mixture contained (final volume of 1 ml in a disposable cuvette) 50 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 0.1 mM NADH, 5 mM DTT, 1 mM Fru-6-P, 0.45 U (unit of enzyme activity) of aldolase, 0.6 U of glycerol-3-phosphate dehydrogenase, 1.8 U of triosephosphate isomerase and 10 mU of PP_i-PF1K. The accessory enzymes were purchased from Roche (Indianapolis, IN). PP_i-PF1K was either from Sigma or purified from a potato tuber according to the conventional method.³⁸ The purified PP_i-PF1K in 40% (v/v) glycerol with protease inhibitors (E64 and Pepstatin from Roche) stored at -20 °C maintained its activity for longer than 1 month. The reaction was started by adding 0.5 mM sodium pyrophosphate. Initial rates of decrease in absorbance at 340 nm were corrected for the rate of the control reaction in which no Fru-2,6-P₂ was present.

The initial velocities were fitted to one of the following equations ((1), competitive; (2), noncompetitive; (3), uncompetitive inhibition) by the program reported by Cleland,³⁹ in which K_{is} and K_{ii} are the dissociation constants for the inhibitor from E•S and E•S•I complexes, respectively. V is the maximal velocity, A is the substrate

concentration, I is the inhibitor concentration, and K is the Michaelis–Menten constant.

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A} \quad (1)$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (2)$$

$$v = \frac{VA}{K + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (3)$$

Preparation and crystallization of PFKFB3 and the mutant proteins

Site-directed mutagenesis of 2-Kase was made using QuickChange PCR (Stratagene, La Jolla, CA) according to the manufacturer's protocol and confirmed by DNA sequencing. The oligonucleotides for mutagenic PCR were synthesized from Invitrogen (Carlsbad, CA). Other procedures of DNA manipulation were according to conventional standard methods. The mutant proteins were over-expressed and purified by the methods used for the wild-type.⁵ Preparation of the protein sample and its crystallization was performed as described.⁵ The His₆-tagged human inducible bifunctional enzyme was expressed in *Escherichia coli* BL21(DE3)pLysS and purified using Ni-NTA affinity columns and the N-terminal His₆ tag was removed by treatment with thrombin using a standard protocol. The final purification was performed using Mono Q anion-exchange chromatography and the resulting pure protein was kept, after concentrating to 8 mg ml⁻¹ protein, in 20 mM Tris–HCl (pH 8.0), 10 mM NaP_i, 0.05 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM ADP, 5% glycerol, 0.2 mM Fru-6-P. Crystals were prepared by the sitting-drop, vapor-diffusion method with a 1:1 (v/v) mixture of the protein sample with a reservoir solution of 50 mM Tris–HCl (pH 7.5), 20–25% ethylene glycol, 12% (v/v) dioxane, 5% glycerol, and 12% (w/v) polyethylene glycol 4000. Crystals with a size of 0.2 mm × 0.4 mm × 0.5 mm grew in two to three weeks.

Diffraction data collection and processing

Crystals were soaked with cryoprotectant solutions for 0.5 to 2 h to allow binding of the targeted ligands before cryogenic data collections.⁵ Depending on the experimental aims, cryoprotectant solutions (a crystallization reservoir solution enriched with 35% ethylene glycol) were enriched with either 0.2 mM AMPPCP•Mg²⁺ and Fru-6-P, or PEP. Both the complexes of PFKFB3•AMPPCP•Fru-6-P and PFKFB3•ADP•PEP were obtained by soaking the PFKFB3 native crystals with solutions containing the target ligands. A soaked crystal was flash-frozen at 100 K using an Oxford cryo-device and kept at the same temperature during data collections. The diffraction data were collected at The Gulf Coast Consortium Protein Crystallography Beamline (PX1) in The Center for Advanced Microstructures and Devices (CAMD), Louisiana State University, Baton Rouge, LA. The X-ray source wavelength was 1.3808 Å. The data recorded on a Mar 165 mm CCD detector were integrated, merged, and scaled using HKL2000.⁴⁰ Statistics of the diffraction data and structure

refinement are summarized in Table 1. The crystals belong to space group *P*6₅22 with similar cell dimensions varied insignificantly by the experimental liganding conditions.

Structure determination and refinement

The reduced data were formatted for the program suites of CCP4⁴¹ and CNS,⁴² and 10% of the data were marked for free *R*-factor measurements in subsequent structure refinements. To ensure a freedom to structural refinements, the indices of reflections in the free data were kept as those of the structure of the PFKFB3•ADP•EDTA complex (PDB accession code 2AXN).⁵ The structures of the two different complexes of PFKFB3•AMPPCP•Fru-6-P and PFKFB3•ADP•PEP were determined by molecular replacement using the first PFKFB3 structure (PDB accession code 2AXN)⁵ as the starting model after stripping all the included ligand and solvent molecules. The molecular replacement solutions were readily achieved using AMoRE⁴³ implemented in the CCP4 suite.⁴¹ The initial model went through iterated cycles of manual model rebuilding using the program O,⁴⁴ and refinement using CNS.⁴² Binding of the included ligands was confirmed, referring to the $|F_o| - |F_c|$ omit maps that were generated, when $R_{\text{crys}}/R_{\text{free}}$ reached 0.28/0.30 or below. Referring to these maps, AMPPCP, Mg²⁺, Fru-6-P, or PEP was incorporated into the corresponding complex models. As summarized in Table 1, the final model of the PFKFB3•AMPPCP•Fru-6-P complex has $R_{\text{free}}/R_{\text{crys}}$ of 0.264/0.224 using a total of 3880 scatterers, including solvent molecules, against all available 21,941 reflections in the resolution range of 30.0–2.7 Å. Because of the limited resolution, only the group *B*-factor refinement was performed for this structure. The structure contains a total of 433 amino acid residues of the full-length protein of 520 residues. As in the PFKFB3•ADP•EDTA complex,⁵ the C terminus (residues 446–520) is mostly disordered.

The structure of the PFKFB3•ADP•PEP complex was determined in a similar way but the restrained individual *B*-factor refinement was made. The final structure has $R_{\text{free}}/R_{\text{crys}}$ of 0.243/0.214 using a total of 4007 scatterers, including solvent molecules, against all available 38,937 reflections in the resolution range of 30.0–2.25 Å. The PFKFB3•ADP•PEP structure contains a total of 449 amino acid residues of the full length protein of 520 residues. For both structures, more than 89% of the residues are in the most favored region, 9.5% in the additional region, and the rest in the generously allowed region in the Ramachandran plots. The structure refinement statistics are summarized in Table 1.

In all PFKFB3-related structures, K204, E380, C386, V226, E330, and K79 are always in the generously allowed region in the Ramachandran plots. Except for K79, all the rest are very well defined by electron density in all structures, although all are located in the loop regions. For all PFKFB3 complex structures, the protein *B*-factor is higher than that of the ligands. The reason is that all this structures includes the N or C-terminal 28–45 residues that are not connected to the core domains. Although their electron density is defined well enough to assign the structure, their *B*-factors are much higher than those of the core regions. If only the core domains are counted, the protein *B*-factors are lower than those of ligands.

Protein Data Bank, accession codes

The atomic coordinates and structure factors of the PFKFB3•AMPPCP•Fru-6-P complex (PDB accession code

2DWP) and PFKFB3•ADP•PEP complex (PDB accession code 2DWO) have been deposited in the RCSB Protein Data Bank.

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