Crystal Structure of the Hypoxia-inducible Form of 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3)

A POSSIBLE NEW TARGET FOR CANCER THERAPY*

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The hypoxia-inducible form of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) plays a crucial role in the progression of cancerous cells by enabling their glycolytic pathways even under severe hypoxic conditions. To understand its structural architecture and to provide a molecular scaffold for the design of new cancer therapeutics, the crystal structure of the human form was determined. The structure at 2.1 Å resolution shows that the overall folding and functional dimerization are very similar to those of the liver (PFKFB1) and testis (PFKFB4) forms, as expected from sequence homology. However, in this structure, the N-terminal regulatory domain is revealed for the first time among the PFKFB isoforms. With a β-hairpin structure, the N terminus interacts with the 2-Pase domain to secure binding of fructose-6-phosphate to the active pocket, slowing down the release of fructose-6-phosphate from the phosphoenzyme intermediate product complex. The C-terminal regulatory domain is mostly disordered, leaving the active pocket of the fructose-2,6-bisphosphatase domain wide open. The active pocket of the 6-phosphofructo-2-kinase domain has a more rigid conformation, allowing independent bindings of substrates, fructose-6-phosphate and ATP, with higher affinities than other isoforms. Intriguingly, the structure shows an EDTA molecule bound to the fructose-6-phosphate site of the 6-phosphofructo-2-kinase active pocket despite its unfavorable liganding concentration, suggesting a high affinity. EDTA is not removable from the site with fructose-6-P alone but is with both ATP and fructose-6-P or with fructose-2,6-bisphosphate. This finding suggests that a molecule in which EDTA is covalently linked to ADP is a good starting molecule for the development of new cancer-therapeutic molecules.

Mammalian tissues have evolved to carry out various functions under different physiological conditions. Such differentiation has naturally caused the tissue cells to have different optima for their energy metabolism. The bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB, 2-Kase/2-Pase), plays a key role in such tissue-specific energy metabolism by controlling glucose utilization. Because this enzyme controls the cellular concentration of fructose-2,6-bisphosphate (Fru-2,6-P₂), which is the most potent allosteric activator of phosphofructokinase, the rate-limiting enzyme of glycolysis, PFKFB eventually controls the glycolytic rate (1–3). The bifunctional enzyme acutely controls the concentration of Fru-2,6-P₂ by modulating the two mutually exclusive catalytic activities of Fru-2,6-P₂ synthesis (2-Kase) and hydrolysis (2-Pase) that reside in the two separate domains (4, 5). The two activities 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 (6–9).

To optimize glucose utilization for physiological roles, every mammalian cell is equipped with one of four PFKFB isoforms, the liver, heart, testis, and inducible forms that are encoded by distinct genes (10–14). Although these isoforms share high “2-Kase/2-Pase core” sequence homology (85%), each isoform has different kinetic properties and responds differently to upstream regulatory signals. This is a function of the highly divergent N- and C-terminal regulatory domains and the sequence differences in the second shell residues surrounding the active sites of the catalytic domains (11). Unlike the other tissue-specific isoforms, the inducible form is ubiquitously expressed by hypoxic stress (10, 15) and found to be identical to the forms previously isolated from placenta (16), pancreatic β-islet (11), or brain (17). The inducible form has an uncharacteristically high and predominant 2-Kase activity with a 2-Kase/2-Pase activity ratio larger than 700 (18, 19). This is contrast to the much lower ratios of the liver, testis, and heart forms of 1.5, 4, and 80, respectively (1, 11). Phosphorylation of the inducible form on Ser-460 by PKA, or AMP-dependent protein kinase, increases the ratio to >3000 (8, 19–22).

Recent studies show that the inducible form is also frequently found in cancer cells (10, 22, 23). Proliferating cancer cells, which have a higher dependence on glucose for their metabolic needs, obtain ATP from glycolysis rather than by oxidative phosphorylation even under aerobic conditions and, thus, show a much higher rate of glycolysis than normal cells (24). In addition, the severe hypoxic stress under which the cancer cells proliferate inside the mass allows no other energy metabolic pathway but glycolysis. Metabolic adaptation to hypoxia is initiated by changes in gene expression patterns triggered by anaerobic activation of hypoxia-inducible factor 1 (HIF-1), and the gene (pfkfb3) for the inducible bifunctional enzyme is one of the most immediate targets of transcriptional activation by HIF-1 (15, 25–27). The extremely high 2-Kase activity of the inducible PFKFB provides the impetus for vigorous glycolysis.

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§ The atomic coordinates and structure factors (code 2AXN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru-2,6-P₂, fructose-2,6-bisphosphate; Fru-6-P, fructose-6-phosphate; 2-Kase, 2-phosphofructo-2-kinase; 2-Pase, fructose-2,6-bisphosphatase; Fru-6-P₂, fructose-6-phosphate; E-P, phosphoenzyme intermediate.
colysis in cancer cells. Recent studies suggest that inhibition of glycolysis to deplete the cellular ATP may serve as an alternative strategy to kill cancer cells that have acquired resistance to traditional drugs. Thus, the inducible PFKFB has been considered as the primary target (26, 28).

We have studied the PFKFB enzyme system for several years and have made significant contributions to understanding the structure/function relationships of the enzyme at a molecular level (29–31). To understand molecular differentiation of the entire PFKFB protein system, which has been the long-term goal of our study, and to provide information on the molecular structure of possible therapeutic targets for cancer, we report here the crystal structure of the human tumor/inducible form of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3).

**MATERIALS AND METHODS**

**Preparation and Crystallization of PFKFB3**—The His-tagged human inducible bifunctional enzyme was expressed in *Escherichia coli* BL21(DE3) pLysS and purified using nickel-affinity columns, and the N-terminal His tag was removed by treatment with thrombin using a standard protocol (19). The final purification was performed using Mono Q anion-exchange chromatography; the resulting pure protein was kept, after concentration to 8 mg ml⁻¹ protein, in pH 8.0, 20 mM Tris-HCl, 10 mM NaPi, 0.05 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM ADP, 5% glycerol, and 0.2 mM Fru-6-P. Crystals were prepared by the sitting drop vapor diffusion of the 1:1 mixture of the protein sample with a mother liquor of 50 mM Tris-HCl, pH 7.5, 20–25% ethylene glycol, 12% dioxane, 5% glycerol, and 12% polyethylene glycol 4000. Crystals in a size of 0.2 × 0.2 × 0.05 mm were grown in 2–3 weeks.

**Data Collection and Processing**—The crystal was soaked with croyo-protectant solutions for 0.5–2 h to remove inorganic phosphate and to incorporate the targeted ligands. A typical croyo-protectant solution is pH 7.5, 20 mM Tris-HCl buffer enriched with 35% ethylene glycol and 1.0 mM Fru-2,6-P₂. A soaked crystal was flash frozen at 100 K using an Oxford cryo-device and kept at the same temperature during data collection.

**Structure Determination and Refinement**—The structure of PFKFB3 complexed with ADP and EDTA was determined first by molecular replacement using AMoRE (35) implemented in the CCP4 suite (33). A single polyalanine form of the liver structure (Protein Data Bank accession code 1K6M) (31) was used as a search model. An AMoRE rotation and subsequent translation search was performed using a data subset between 15.0 and 4.0 Å. The molecular replacement search as well as the measurement of solvent fraction suggested a single protein molecule in an asymmetric unit. A good molecular replacement solution was found to show an R-factor of 0.52 and a correlation factor of 0.58. Assignment of the side chains was made using program O (36). The initial model went through iterative cycles of manual model rebuilding using O program and refinement at 2.1 Å using CNS (34). When R_cryst/R_free reached 0.27/0.29 or below, the ligand molecules were built into the model, referring to the |F_cal| – |F_obs| omit maps. ADP and the 2-Pase-bound Fru-6-P were incorporated first, and EDTA was added after mass spectrometric confirmation of the crystal. As summarized in Table 1, the final model has R_free/R_cryst of 0.233/0.209 using a total of 4,085 scatterers, including solvent molecules, against all available 44,651 reflections in the resolution range of 30.0–2.1 Å. The structure contains a total of 452 protein residues of the full-length protein of 520 residues.

The subsequent structure of PFKFB3 complexed with ADP and Fru-2,6-P₂ was determined by using the structure of the protein-ADP-EDTA complex after stripping the ligands and solvents as the initial model and by following the structural refinements procedures similar to that used for the structure of the protein-ADP-EDTA complex. To guarantee a freedom to structural refinements, the indices of reflections in the free data were kept as those of the protein-ADP-EDTA complex. The final structure has R_free/R_cryst of 0.262/0.214 using a total of 3,945 scatterers, including solvent molecules, against all available 28,580 reflections in the resolution range of 30.0–2.5 Å. The structure contains a total of 449 protein residues of the full-length protein of 520 residues. The refinement statistics are summarized in Table 1.

**RESULTS AND DISCUSSION**

**Overall Structure**—The PFKFB3 protein was crystallized under conditions similar to those for the liver form (31). The structures from the two different liganding conditions were determined by molecular replacement using the liver structure as a start model. The two different liganding conditions used were ADP and fructose-6-phosphate (Fru-6-P) and ADP and Fru-2,6-P₂, and the resulting resolutions of the final structures were 2.1 and 2.5 Å, respectively. Among all the structure-assigned residues, 89% are in the most favored regions in a Ramachandran plot, 9.5% in the additional allowed region, and the rest in the generously allowed region. Details of the crystallographic statistics are summarized in Table 1. Most descriptions of the structure below are according to the structure determined to 2.1 Å resolution, unless spe-

**TABLE 1**

Statistics of reflection data and structure refinements

<table>
<thead>
<tr>
<th>Ligand</th>
<th>PFKFB3-ADP-EDTA</th>
<th>PFKFB3-ADP-Fru-2,6-P₂</th>
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</thead>
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<tr>
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<td>P6₁,22</td>
</tr>
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<tr>
<td>Resolution range (Å)</td>
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<td>30.0–2.5</td>
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<td>28,580</td>
</tr>
<tr>
<td>Completeness (%)</td>
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<td>99.7 (97.8)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>9.6 (9.3)</td>
<td>13.9 (9.2)</td>
</tr>
<tr>
<td>R(int)</td>
<td>16.9 (1.5)</td>
<td>15.3 (1.9)</td>
</tr>
<tr>
<td>R_cryst</td>
<td>0.107 (0.687)</td>
<td>0.099 (0.535)</td>
</tr>
<tr>
<td>R_free</td>
<td>0.209</td>
<td>0.214</td>
</tr>
<tr>
<td>ΔRmerge</td>
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<td>0.262</td>
</tr>
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<td>No. of amino acids</td>
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<td>449</td>
</tr>
<tr>
<td>No. of protein atoms</td>
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<td>3,658</td>
</tr>
<tr>
<td>No. of hetero atoms</td>
<td>63</td>
<td>66</td>
</tr>
<tr>
<td>No. of waters</td>
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</tr>
<tr>
<td>Angles (°)</td>
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<td>1.3</td>
</tr>
<tr>
<td>Dihedral angles (°)</td>
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<td>22.2</td>
</tr>
</tbody>
</table>

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Crystal Structure of PFKFB3

As predicted from the high sequence homology (85%) between the PFKFB isoforms, the folding of the inducible form is very similar to those of the tests and liver forms (31, 37). As shown in Fig. 1a, a PFKFB-typical head-to-head functional dimerization is also conserved.

However, the inducible form also has structural features uniquely different from the other isoforms. The conformations of the substrate loops in the 2-Kase domain are different from those of other isoforms (31, 37), providing a structural rationale for the higher 2-Kase activity. The structure of the N-terminal regulatory domain is revealed: the N terminus binds to the 2-Pase domain to cause a local conformational change in the active pocket to enhance inhibitory binding of product. Most strikingly, an EDTA molecule was found at the Fru-6-P site of the active pocket of 2-Kase. Detailed descriptions of these structural features of the inducible form will be made in following sections.

**Autoregulation of the 2-Pase by the N-terminal Domain**—This is the first time the structure of the regulatory N terminus (residues 2–28 of 31 residues) has been revealed among any of the solved PFKFB structures. As shown in Fig. 1, a and b, residues 4–15 form a β-hairpin structure, and the rest serve as an arm connecting the hairpin to the 2-Kase. The turn of this hairpin makes a contact with its own 2-Pase domain and causes a global rotation of the 2-Pase domain from its relative position in the liver form (data not shown). Although the domain twist is interesting, its functional effect is not clear.

On the other hand, the contacting area of the 2-Pase domain is functionally very sensitive because the residues critical for binding of both product and substrate are located in close proximity (Fig. 1c). The three key residues, Arg-347, Lys-351, and Tyr-362, which interact with the 6-phosphate moiety (6-P) of substrate (Fru-2,6-P2) and product (Fru-6-P) are located in this area (38, 39). Binding of the N terminus to this area is mediated by salt bridges and/or hydrogen bonds between Arg-8 and Glu-342, between the main chain of Val-9 and that of Tyr-354, between Glu-10 and Glu-363 and Arg-355, and between Lys-11 and the main chain of Arg-355. As a result, the β-strand where Tyr-354, Arg-355, and Tyr-356 are located moves deep inside by more than 1 Å; as a result, a local network of side chain interactions is reorganized when compared with that of the liver form. Tyr-354 makes a hydrogen bond to Glu-343 and Tyr-356 to Arg-347. Consequently, the interaction of Arg-347 with 6-P of the bound Fru-6-P becomes stronger with a distance shortened by 0.7 Å. Similarly, the distances from 6-P to Lys-356 and Tyr-362 are shortened by 0.7 and 0.4 Å, respectively. This may cause a substantial increase in affinities for both substrate and product.

Because of sequence difference from other isoforms, the inducible form has a serine instead of an arginine at the 302nd residue. The corresponding Arg-307 in the liver form and all other isoforms has dual functions by providing a positive charge for the 2-phosphate moiety (2-P) of substrate: facilitating substrate binding and stabilizing the phosphoenzyme intermediate (E-P) during its breakdown (40). Thus, the inducible PFKFB is doubly compromised by the loss of arginine at this position; its affinity for substrate is decreased, and the rate of E-P breakdown is drastically slowed. Yet, binding of the product, Fru-6-P, which is a potent non-competitive inhibitor of the reaction, is not affected. Site-specific mutagenesis of the liver 2-Pase showed similar results, and the current structure supports the observation (40). Thus, the net result of the loss of arginine in the inducible PFKFB is that its 2-Pase reaction proceeds more slowly than the E-P-Fru-6-P complex state. This was kinetically observed in a recent study in which the arginine residue was restored (19). In addition, the interaction with the N terminus mentioned above confers an even tighter binding of Fru-6-P, reducing the activity of the inducible form to negligible rates. Consequently, the N terminus exerts a negative regulatory effect on the 2-Pase activity by enhancing the binding of inhibitor, Fru-6-P, or, more precisely, by slowing down the catalytic rate-limiting step, the release of Fru-6-P from the E-P-Fru-6-P complex. Supporting this hypothesis, the alternative mRNA splicing product of the inducible PFKFB, in which the N terminus is naturally deleted, has a 7-fold higher 2-Pase activity (19).
Substrate Binding Loops of the 2-Kase Domain—The 2-Kase domain has binding sites for the two substrates, ATP and Fru-6-P, the binding of which are sensitive to the conformations of the two outer lobes known as the ATP loop (residues 168–179) and the Fru-6-P loop (residues 72–84) that surround the whole active pocket (31, 41). In the testis form, the two loops are structurally coupled to each other through two hydrogen bonds donated by Arg-181. Upon binding of ATP, the last turn of helix H9251 unwinds to allow Lys-172 to interact with H9253 -phosphate. This conformational change is transmitted to the Fru-6-P loop via Arg-181 such that the Fru-6-P loop has a new conformation. Fru-6-P can bind only to this new conformation (41). Thus, binding of Fru-6-P is dependent on that of ATP in the testis form in an ordered fashion. However, the native liver form with a cysteine instead of an arginine has the two loops decoupled from each other. As a result, their conformations are different from those in the testis form, and the two substrates bind independently from each other (31).

The inducible form also has a cysteine instead of an arginine, and thus the two loops are decoupled like in the liver form, allowing the independent binding of substrate. In addition, as shown in Fig. 2a, sequence differences in the residues of N178d and E181k provide new interactions necessary to form an additional helix turn to the end of the ATP loop. The substitution of Gln-80 with serine and an added Lys-79 change the local conformation of the Fru-6-P loop to a helix turn; as a result, Tyr-81 makes a hydrogen bond to the main body of the following helix through Asp-185.

The sequence differences in these loop regions add helix turns to the two substrate loops, decreasing the conformational flexibilities of these loops compared with the coil structures of the other forms. The restricted conformations of the two loops appear to enhance binding of substrates.
Crystal Structure of PFKFB3

**ATP Binding and the 2-Pase—**As shown in Fig. 2b, because of the sequence differences of S162e and M165r from the liver form, the solvent-exposed α5 moves downward and eventually creates a hydrogen bond between Glu-166 and Ala-423, which does not exist in other isoforms. Consequently, the conformation of the unwound last turn of α5 is kept stable and pulls a β-hairpin (residues 415–432) from the 2-Pase domain toward α5. The hairpin has Tyr-424, which is the only residue from the 2-Pase domain directly involved in the 2-Kase reaction by interacting with ATP. With help from the water-mediated hydrogen bond between Lys-52 and Glu-72, which intercalates the phenyl ring of Tyr-424 between Lys-52 and Val-167, the interaction between Tyr-424 and the bound ADP is firmer with the distance shortened by 0.5 Å. This may increase the affinity of the 2-Kase for ATP.

As described above, the different amino acid sequences introduced into the inducible form contribute to the conformations of the two substrate loops and the other structural elements required for substrate bindings. Together, they provide a structural basis for better affinities for substrates and, ultimately, the highest 2-Kase activity among the PFKFB isoforms.

**Binding of EDTA to the Fru-6-P Site of 2-Kase—**Despite continuous efforts for the last several years, structural characterization of the Fru-6-P site in the 2-Kase active pocket has never been successful. Structural information on this site is very important for understanding of the catalytic mechanism carried out by the 2-Kase of all PFKFB isoforms. As a trial to define the site, the crystals of the inducible form PFKFB were soaked with a cryoprotectant containing 0.2 mM Fru-6-P before data collection. When the| − | map was generated (Fig. 3a, top), the putative Fru-6-P site was found to be occupied by an EDTA molecule. It was surprising because the Fru-6-P concentration was 3-fold higher than that of EDTA and because EDTA has never been reported to compete with Fru-6-P for the 2-Kase. Moreover, almost all experimental conditions for the 2-Kase assay to date have at least 0.05 mM EDTA, the same concentration that we used. These circumstances cultivated suspicion about the functional nature of this site. To clear the ambiguity, the structures from the crystals soaked with different ligands were summarized in Table 2.

**TABLE 2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>EDTA Ligand</th>
<th>Distance (Å)</th>
<th>Protein</th>
<th>Fru-2,6-P₂ Ligand</th>
<th>Distance (Å)</th>
</tr>
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<tbody>
<tr>
<td>Lys-47</td>
<td>NZ</td>
<td>O5</td>
<td>Arg-74</td>
<td>NH₂</td>
<td>O2P</td>
</tr>
<tr>
<td>Arg-74</td>
<td>NE</td>
<td>O₄</td>
<td>Arg-98</td>
<td>NH₂</td>
<td>O₄P</td>
</tr>
<tr>
<td>Arg-75</td>
<td>NH₁</td>
<td>O₇</td>
<td>Arg-132</td>
<td>NH₁</td>
<td>O₃P</td>
</tr>
<tr>
<td>Arg-81</td>
<td>NH₁</td>
<td>O₈</td>
<td>Arg-189</td>
<td>NE</td>
<td>O₁</td>
</tr>
<tr>
<td>Thr-126</td>
<td>OG₁</td>
<td>O₉</td>
<td>Thr-126</td>
<td>OG₁</td>
<td>O₆P</td>
</tr>
<tr>
<td>N</td>
<td>N</td>
<td>O₆</td>
<td>Tyr-193</td>
<td>OH</td>
<td>O₄P</td>
</tr>
</tbody>
</table>

As shown in Fig. 3a, the two omit maps are clearly different and suggest two different molecules. Binding of EDTA and Fru-2,6-P₂ were subsequently confirmed by mass spectrometric analysis of the soaked crystals (data not shown). When the final structures were compared (Fig. 2, b and c), it was found that Arg-74, Arg-98, and Thr-126 are introduced in this report. The omit map from the Fru-2,6-P₂-soaked crystals (data not shown). When the final structures were compared in the figures, the two molecules occupy nearly the same place, a pocket right next to the ADP site. Finally, it was concluded that EDTA binds to the Fru-6-P site. Interactions between the protein residues and the two ligands are summarized in Table 2.

As shown in Fig. 3b and summarized in Table 2, seven of eight oxygen atoms in EDTA are recruited for salt bridges or hydrogen bonds to the protein residues, which are all positively charged except for Thr-126. To accommodate these interactions with protein, EDTA has an extended conformation. As indicated by the interaction distances in Table 2, the interactions between EDTA and protein are very tight, explaining the unexpectedly high affinity of EDTA. As shown in Fig. 3, ADP is located right next to EDTA with an approximate distance of 3 Å, which is shorter than two average covalent bonds. This proximity suggests a molecular scaffold of the potential inhibitors for the 2-Kase activity of the inducible bifunctional enzyme. As mentioned in the Introduction, the inducible PFKFB is one of the major driving force molecules responsible for tumorigenesis. Suppression of vigorous glycolysis, a hallmark of oncogenic cells, has been suggested as an effective alternative to traditional cancer therapies, and genetic disruption of the inducible PFKFB has been observed to induce deaths of cancerous cells (10, 22, 26, 28). Therefore, an efficient inhibitor for the 2-Kase activity of the inducible PFKFB can serve as a new therapeutic molecule for cancer. Based on the current structure, we suggest a scaffold of the inhibitor new synthetic molecules to be a conjugate molecule in which one acyl group of EDTA is covalently linked to β-phosphate of ADP.

**Biological Significance—**Among the four PFKFB isoforms, only the structures of the liver and testis forms have been solved (31, 37), and with the results presented here, the inducible form may be added to the list. The previous structural studies have made great contributions toward understanding of the PFKFB protein at the molecular level. Nonetheless, catalytic mechanism of their 2-Kinase reaction is not very well understood, because structural characterization of the Fru-6-P pocket has never been successful. In this report, we provided information on the interactions between the protein and Fru-6-P. To provide long-awaited information on the 2-Kinase reaction, a subsequent structure/function study is being made.

Finding of the N terminus is particularly significant because it seems to exert self inhibition on the 2-Pase, which is more distantly located than the 2-Kase. The previous functional studies of the liver enzyme have suggested that the N terminus may interact with the neighboring 2-Kase domain to enhance Fru-6-P binding (42). Based on this observation, it was expected that the N terminus of PFKFB3 might also be located near the 2-Kase domain. However, the sequences for the hairpin structure (residues 4–15) appear to be well conserved among the inducible, liver, and testis forms, suggesting that N-terminal sequences of the liver and testis forms may also form hairpin structures to exert their regulatory effects. This is consistent with early kinetic data on enhanced 2-Pase activity observed with N-terminal phosphorylation (43). However, because the residues connecting the hairpin to the 2-Kinase
domains are highly divergent among the isoforms, it is unpredictable where their N termini would bind.

Together with our previous structural studies (31), the current study suggests that a small number of sequence variations in the catalytic domains cause differences in the substrate loop interactions and that the variable N- and C-terminal regulatory domains function as intrinsic inhibitors of the catalytic domains. Combination of these allows the four tissue isoforms of PFKFB to have different kinetic properties and regulatory mechanisms. Putting aside the C-terminal domains, which either are disordered (the inducible form) or cover the 2-Pase active pocket (the liver and testis forms), the varied loop interactions and/or the N terminus produce differing binding affinities and release rates of substrates or products during the catalytic steps. Mechanistically, these functional effects are made by strengthening or weakening the interactions between the key residues and substrates/products. To achieve this, they affect the secondary shell residues, which are located next to the key residues to determine electrochemical environments of the catalytic reactions.

Compared with alterations in the primary components such as con-stellations of catalytic residues that can easily cause orders of magnitude differences in kinetic properties (44), variations in the secondary components are more suitable for modest kinetic differences in a range of 2- to 20-fold, which is comparable with the differences between the PFKFB isoforms, and thus are employed as a central scheme of the molecular reactions.

Crystal Structure of PFKFB3

Binding of EDTA to PFKFB3 raises a possibility that EDTA might have induced a non-physiological conformation of PFKFB3. The suggestion could be ruled out by two observations. First, PFKFB3 can be crystallized without EDTA. Second, when the crystal was soaked with ATP and Fru-6-P, Fru-2,6-P2 was synthesized and found in the structure (data not shown), indicating that the conformation in the presence of EDTA is suitable for the physiological function of PFKFB3.

The inducible PFKFB is one of the major metabolic effectors that facilitate cancer progression. Suppression of glycolysis has been frequently suggested as an effective alternative to traditional cancer ther-apies; accordingly, the inducible PFKFB has been considered as one of the primary targets of such approaches. The discovery of EDTA at the Fru-6-P site provides a molecular paradigm of a new therapeutic molecule that targets the 2-Kase activity of the inducible PFKFB for cancer treatments. A suggested starter molecule resembles EDTA covalently linked to the β-phosphate of ADP.

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