ADP-dependent 6-Phosphofructokinase from Pyrococcus horikoshii OT3

STRUCTURE DETERMINATION AND BIOCHEMICAL CHARACTERIZATION OF PH1645

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Some hyperthermophilic archaea use a modified glycolytic pathway that employs an ADP-dependent glucokinase (ADP-GK) and an ADP-dependent phosphofructokinase (ADP-PFK) or, in the case of Methanococcus jannaschii, a bifunctional ADP-dependent glucophosphofructokinase (ADP-GK/PFK). The crystal structures of three ADP-GKs have been determined. However, there is no structural information available for ADP-PFKs or the ADP-GK/PFK. Here, we present the first crystal structure of an ADP-PFK from Pyrococcus horikoshii OT3 (PhPFK) in both apo- and AMP-bound forms determined to 2.0-Å and 1.9-Å resolution, respectively, along with biochemical characterization of the enzyme. The overall structure of PhPFK maintains a similar large and small α/β domain structure seen in the ADP-GK structures. A large conformational change accompanies binding of phosphoryl donor, acceptor, or both, in all members of the ribokinase superfAMILY characterized thus far, which is believed to be critical to enzyme function. Surprisingly, no such conformational change was observed in the AMP-bound PhPFK structure compared with the apo structure. Through comprehensive site-directed mutagenesis of the substrate binding pocket we identified residues that were critical for both substrate recognition and the phosphotransfer reaction. The catalytic residues and many of the substrate binding residues are conserved between PhPFK and ADP-GKs; however, four key residues differ in the sugar-binding pocket, which we have shown determine the sugar-binding specificity. Using these results we were able to engineer a mutant PhPFK that mimics the ADP-GK/PFK and is able to phosphorylate both fructose 6-phosphate and glucose.

Glycolysis is a central and highly conserved metabolic pathway in all three domains of life. However, the Embden-Meyerhof glycolytic pathway of some hyperthermophilic archaea displays distinct differences from the classical pathway. Glyceraldehyde-3-phosphate is converted to 3-phospho (3P)-glycerate by glyceraldehyde-3-phosphate ferredoxin oxidoreductase in a single step instead of using glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase in the classical two-step reaction mechanism (1, 2). Also, the classical ATP-dependent glucokinases (ATP-GKs)* and phosphofructokinases (ATP-PFKs) are replaced with novel ADP-dependent glucokinases and ADP-dependent phosphofructokinases or, in the case of Methanococcus jannaschii, a bifunctional ADP-dependent gluco-/phosphofructokinase (ADP-GK/PFK) (3–7).

PFKs convert fructose 6-phosphate (Fru-6-P) to fructose 1,6-bisphosphate. This is an early step in the Embden-Meyerhof pathway and therefore represents a critical control point for the entire pathway. Sequence and structural characterization classify PFKs into two convergent protein families (8): the PFK-A family and the ribokinase superfamily, which includes the PFK-B family and ADP-GK/ADP-PFK family. The PFK-A family includes both allosterically regulated ATP-dependent enzymes found in a variety of eukarya and bacteria and non-allosterically regulated PP₂-dependent enzymes found in all three domains of life (9, 10). The PFK-B family is a diverse family of ATP-dependent carbohydrate and pyrimidine kinases that is also present in all three domains of life. In general, ATP-PFKs from the PFK-B family can be differentiated from PFK-A ATP-PFKs by their lack of allosteric regulation, although the minor ATP-PFK from Escherichia coli, Pfk-2, is allosterically regulated by MgATP (12).

Several crystal structures of the PFK-A family PFKs have been reported. The best studied are the tetrameric PFKs from E. coli and Bacillus stearothermophilus (13, 14). Both contain subunits that consist of a large and a small 3-layered αβα sandwich domain (13, 14). The large domain binds to ATP and the small domain binds to Fru-6-P (13, 14). Recently the crystal structure of Pfk-2, a member of the PFK-B family, was solved (15). It is composed of two domains, a large 3-layered αβα sandwich
domain, similar to that seen in the PFK-A family protein structure mentioned above, and a smaller four-stranded β-sheet domain (15).

To date ADP-PFKs have been reported as non-regulated enzymes with Michaelis-Menten kinetics. This part of glycolysis is particularly interesting because with the presence of PFKs and fructose-1,6-bisphosphatase there is the possibility to produce futile cycling resulting in a net hydrolysis of nucleotide (ADP in the case of thermococcales) which, of course is undesirable because it would uncouple the metabolism. E. coli has overcome this problem by using a strong MgATP-induced inhibition when Fru-6-P is low in both of its PFKs: Pfk-1 from the PFK-A family and Pfk-2 from the ribokinase superfamily (16, 17). On the other hand, the archaea with the modified Embden-Meyerhof pathway seems to control this issue at the transcriptional level as, depending on the growth medium, either the production of PFK or fructose-1,6-bisphosphatase is turned off (18). In this way, regulation of PFK is not needed.

The crystal structures of the ADP-GK from Pyrococcus horikoshii (PhGK), the ADP-GK from Thermococcus litoralis (TlGK) bound to ADP, and the ADP-GK from Pyrococcus furiosus (PfGK) in complex with AMP and glucose have been reported, although there are no crystal structures of ADP-PFK or the bifunctional ADP-GK/PFK (19–21). Despite low sequence identity between ADP-GKs and other kinases (e.g. PhGK shares 11 and 12% identity with E. coli ribokinase and human adenosine kinase, respectively) the fold is similar to that of ribokinases (19). As a result, ADP-GKs have been classified as members of the ribokinase superfamily (19). Overall, ADP-GK structures are composed of a large and a small α/β domain (19–21). The ADP binding pocket is found on the surface of the large domain (19–21). Residues responsible for glucose binding are found on the surface of both the large and small domains adjacent to the ADP-binding pocket (19–21). Upon glucose binding, the protein undergoes a conformational change whereby the large and small domains close in creating the active-site pocket (20). Interestingly, the unique ADP binding pocket of ADP-GKs recognizes the α- and β-phosphate of the ADP in an almost identical manner to how the β- and γ-phosphate of ATP are recognized in ATP-dependent kinases of the ribokinase superfamily (19).

Here, we report the first structure of an ADP-PFK, alone and in complex with AMP, together with the biochemical characterization of the kinetic properties and substrate specificities. Moreover, we carried out site-directed mutagenesis in the sugar-binding pocket and active site of PhPFK and identified residues that are critical for PFK activity and for distinguishing between glucose and Fru-6-P.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Protein Purification—The open reading frame for PhPFK (PH1645) was amplified by PCR and inserted between the NdeI and BamHI restriction sites of a modified pET-15b expression vector (Novagen) (vector p11) as previously described (22). This construct generated an N-terminal hexahistidine tag joined to the PhPFK protein by the TEV protease recognition site (ENLYFQ ↓ G). Recombinant wild type and mutant (see below) native and selenomethionine-labeled PhPFK proteins were expressed in BL21(DE3) and DL41(DE3) E. coli cells (Novagen), respectively. Protein was purified using nickel-nitritotriacetic acid affinity and size exclusion chromatographies. For more details see supplemental materials.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol using the PhPFK expression vector described above as the template. See Table 1 for the list of mutations made.

Protein Crystallization—Protein crystals of apo-PhPFK were generated through hanging drop vapor diffusion at 21 °C by mixing 2 μl of protein solution (10 mg/ml) with 2 μl of well solution consisting of 22% PEG 4000, 0.1 M Tris-HCl, pH 8.5, and 0.2 M LiSO₄. Crystals of the PhPFK complex with AMP were obtained by crystallization of the PhPFK D17A protein in the presence of 20% PEG 3350, 0.2 M lithium citrate, 10 mM fructose 6-phosphate, and 5 mM ADP. Prior to data collection, crystals were transferred into Paratone-N and cryo-cooled in a nitrogen gas steam.

X-ray Diffraction, Structure Determination, and Refinement—Apo-PhPFK crystals were placed in a cryoprotectant composed of 15% glycerol added to the crystallization solution and then flash frozen in liquid nitrogen prior to data collection. Multiwavelength anomalous dispersion data were collected at three wavelengths 0.96396, 0.97918, and 0.97943 Å at the Advanced Photon Source (APS, Argonne, IL) beaml ine 19-ID of the Structural Biology Center-CAT with a SBC-3 CCD detector. The data were processed using HKL2000 (23). Data collection and processing statistics are shown in Table 2.

The structure of PhPFK was solved using the multiwavelength anomalous dispersion method. Selenium sites were located using SOLVE (24, 25). Six of the 10 expected selenium sites per asymmetric unit were found. Selenium position refinement, phase calculation, and density modification was performed by SHARP (26). The structural model was built and refined by XFIT, CNS, and Refmac (27, 28). The AMP-bound structure was solved by molecular replacement using Phaser and the model was validated in Coot (29, 30). The final refinement statistics can be found in Table 2.

Determination of Enzyme Activity—PFK activity was assayed spectrophotometrically at 50 °C, by coupling the fructose-1,6-bisphosphate formation to the oxidation of NADH as previously described (3). See supplemental materials for more details.

Substrate Specificity—The phosphoryl group donor specificity of PhPFK was determined by measuring the PFK activity as described above substituting ADP for the following phosphoryl group donors, UDP, IDP, GDP, and CDP. The activity is reported as percentage of the maximum activity obtained with ADP. The divalent cation preference was tested similarly, replacing MgCl₂ in the assay by MgSO₄, MnCl₂, NiSO₄, CaCl₂, or ZnCl₂. 2.5, 4, and 7 mM total metal were used, keeping other components as those used in the standard assay. The activity observed using these metals was reported as units/mg.

Kinetic Parameters—Kinetic parameters were determined at 50 °C by varying the concentrations of Fru-6-P (0 to 2 mM) with saturating ADP concentrations, and vice versa (3). For some
mutants, the range of Fru-6-P concentrations was increased. In all these experiments, MgCl₂ was used as the divalent cation. The data were analyzed using SigmaPlot software (Systat Software, Inc., CA), and fitted to the Michaelis-Menten equation.

RESULTS AND DISCUSSION

Enzymatic Activity and Substrate Specificity of PhPFK—PhPFK is predicted to be an ADP-PFK based on sequence similarity (Fig. 1). In addition, the PhPFK structures align well with the available ADP-GK structures (Fig. 2, C and D). Therefore, to test whether this enzyme is a true ADP-PFK the phosphorylation of Fru-6-P was assayed in the presence of several nucleotides. PFK activity was the highest in the presence of ADP or UDP as the phosphoryl group donor (Fig. 3A). However, PhPFK is also capable of transferring phosphoryl groups from IDP, GDP, and CDP to Fru-6-P but at a significantly slower rate (Fig. 3A). A saturation curve was generated for both ADP and UDP. The calculated $k_{cat} \cdot K_m$ value for UDP was 40 times higher than that of ADP, indicating a strong preference for ADP over UDP as the phosphoryl group donor.

Wild type PhPFK displayed Michaelis-Menten kinetics at 50 °C. The following constants were determined using direct fit: $K_m$ values of 15.2 ± 2.5 and 13.2 ± 2.4 μM for Fru-6-P and ADP, respectively, and a $k_{cat}$ value of 45.5 ± 4.0 s⁻¹. All kinetic parameters are summarized in Table 1 and Fig. 3B. The temperature of these reactions is much lower than the optimal growth temperature of P. horikoshii and because temperature has a significant effect on enzyme kinetics, the constants calculated here may differ from the values at the optimal growth temperature. Unfortunately, the commercially available auxiliary enzymes for this reaction are from mesophilic sources hindering the use of high temperatures in the assay. On the other hand, due to the large amount of mutants characterized in this article the study would be prohibitively longer using a discontinuous assay to measure the kinetic parameters at higher temperatures. Nevertheless, most of the work published for other enzymes from this family uses the same strategy, making the data here directly comparable. The calculated $K_m$ values for Fru-6-P and ADP for wild type PhPFK are significantly lower than those of the ADP-PFK from P. furiosus, 2.3 and 0.11 mM, respectively (7). However, the $K_m$ for Fru-6-P is similar to the $K_m$ obtained for the bifunctional ADP-GK/PFK from M. jannaschii, 9.6 μM. But, the $K_m$ for ADP is still much lower, 0.49 mM (6).

Divalent cations were required for PhPFK activity. Five divalent metal cations were tested (magnesium was tested with two counter ions to discard any effect of the anion) using 2.5, 4, and 7 mM of total metal with 2 mM total ADP and 2 mM total Fru-6-P.
In these conditions, the highest PhPFK activity was obtained with NiSO$_4$ (97 units/mg). The enzyme was also active in the presence of MgCl$_2$, MnCl$_2$, MgSO$_4$, and CaCl$_2$, but no significant activity was detected when the assay was performed in the presence of ZnCl$_2$. An increase in the enzyme activity, concomitantly with the increase in MnCl$_2$ concentration was observed, whereas the opposite effect was observed in the presence of NiSO$_4$. For the other divalent metals, the activity did not change with the cation concentration in the range tested. CaCl$_2$ showed the lowest activity with 75% of the activity measured in the presence of MgCl$_2$. Although it is generally accepted that magnesium is the in vivo preferred metal, the ability of Ni$^{2+}$, Mn$^{2+}$, and to a lower extent Ca$^{2+}$ to support PhPFK activity with a high catalytic rate, suggests that other metals can substitute Mg$^{2+}$.

**Overall Structure**—The apo-PhPFK structure (Fig. 2A) was refined to 2.0-Å resolution with $r = 0.177$ and $R_{free} = 0.226$ (Table 2). The structure contained 450 of the expected 474 residues from the expressed protein construct (Table 2). In the final model, 97.9% of the residues were in favored regions of the Ramachandran plot and none were in the disallowed region (Table 2). PhPFK was crystallized alone and in complex with ADP. The apo structure was solved by the multiwavelength anomalous dispersion method using a selenomethionine derivative and the complex structure was solved by molecular replacement using the apo structure as a model. AMP, instead of ADP, was found in the active site. The AMP-bound structure (Fig. 2B) was refined to a resolution of 1.9 Å with $r = 0.177$ and $R_{free} = 0.228$ (Table 2). Both the apo- and AMP-bound PhPFK crystal structures contained two molecules in the asymmetric unit. For a more detailed discussion of oligomeric state see supplemental materials.

The crystal structure of PhPFK was aligned with PhGK using the DaliLite server (Fig. 2C) (31). PhPFK and PhGK share 31%
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### Table 1

| Enzyme | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_m \) (Fru-6-P) (\( \mu \text{M} \)) | \( k_{\text{cat}}/K_m \) (Fru-6-P) (\( s^{-1} \mu \text{M} \)) | \( K_m \) (ADP) (\( \mu \text{M} \)) | \( k_{\text{cat}}/K_m \) (ADP) (\( s^{-1} \mu \text{M} \)) |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wild type | 45.5 ± 0.3 | 15.2 ± 2.5 | 2.99 × 10\(^{3}\) | 13.2 ± 2.4 | 3.45 × 10\(^{3}\) |
| N15A | 91.1 ± 3.9 | 103 ± 1.4 | 8.84 × 10\(^{2}\) | 23.8 ± 2.6 | 3.83 × 10\(^{2}\) |
| D17A | 1.98 ± 0.0051 | 22600 ± 840 | 8.76 × 10\(^{1}\) | ND | ND |
| A71E | 39.3 ± 0.9 | 3.95 ± 0.2 | 1.72 × 10\(^{1}\) | 13.6 ± 0.6 | 2.82 × 10\(^{1}\) |
| Q797A | 13.0 ± 1.0 | 7.08 ± 0.74 | 1.84 × 10\(^{2}\) | 7.47 ± 0.70 | 1.74 × 10\(^{2}\) |
| K189A | 41.0 ± 6.7 | 6500 ± 1300 | 6.31 × 10\(^{2}\) | 37.9 ± 6.7 | 1.98 × 10\(^{2}\) |
| N160A | 151 ± 16 | 415 ± 13 | 3.64 × 10\(^{2}\) | 72.4 ± 4.9 | 2.09 × 10\(^{1}\) |
| S183E | 91.7 ± 23.6 | 86.5 ± 9 | 1.06 × 10\(^{2}\) | 29.6 ± 2.2 | 3.10 × 10\(^{1}\) |
| R185A | 0.351 ± 0.016 | 318 ± 9.5 | 1.10 × 10\(^{2}\) | 473 ± 24 | 7.42 × 10\(^{1}\) |
| S189A | 25.5 ± 2.6 | 9.02 ± 2.3 | 2.83 × 10\(^{2}\) | 16.0 ± 1.2 | 1.60 × 10\(^{2}\) |
| R191A | 27.4 ± 1.5 | 254.4 ± 26.1 | 1.07 × 10\(^{2}\) | 14.5 ± 1.2 | 1.89 × 10\(^{1}\) |
| R191E | 42.5 ± 1 | 4870 ± 173 | 8.73 × 10\(^{3}\) | 60.5 ± 3.2 | 7.02 × 10\(^{3}\) |
| Q224A | 65.3 ± 2.6 | 38.6 ± 1.3 | 1.69 × 10\(^{2}\) | 29.5 ± 5.0 | 2.21 × 10\(^{2}\) |
| S263A | 64.1 ± 14 | 39.5 ± 7.8 | 1.62 × 10\(^{2}\) | 20.8 ± 3.4 | 3.08 × 10\(^{2}\) |
| D433A | 0.00480 ± 0.00040 | 12.7 ± 4.2 | 3.78 × 10\(^{2}\) | 13.9 ± 3.3 | 3.45 × 10\(^{2}\) |

*ND*, not determined. For the D17A mutant the kinetic parameters for MgADP were not determined because the elevated \( K_m \) value for Fru-6-P hinders reaching the saturating conditions.

### Table 2

Crystallographic data and refinement statistics: \( R_{	ext{sym}} = \Sigma |I(h) − \langle I(h) \rangle|/\Sigma I(h) \), where \( I(h) \) and \( \langle I(h) \rangle \) represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all measurements. Values in parentheses are for the highest resolution shell. PDB codes are 1U2X and 3DRW for apo-PhPFK and its complex with AMP, respectively.

| Structure | Apo-PhPFK | Complex with AMP |
|-----------|-----------|-----------------|
| Space group | P2\(_1\) | P2\(_1\) |
| Unit cell dimensions | \( a = 68.07\) Å, \( b = 99.93\) Å, \( c = 82.58\) Å, \( \beta = 110.38^\circ \) | \( a = 68.5\) Å, \( b = 104.2\) Å, \( c = 78.0\) Å, \( \beta = 105.1^\circ \) |
| Resolution range (Å) | 40.0–2.0 | 41.5–1.9 |
| Unique/free reflections (%) | 96.8% (95.7%) | 98.7% (90.7%) |
| Completeness | 99.2% (98.6%) | 99.7% (90.7%) |
| \( R_{	ext{merge}} \) | 0.08 (0.445) | 0.08 (0.353) |
| \( I/\sigma(I) \) | 21.0 (3.57) | 16.7 (3.25) |
| Redundancy | 6.6 (5.9) | 4.5 (3.9) |
| \( R_{	ext{merge}}/R_{merge} \) (%) | 20.2/24.5 | 17.3/22.1 |
| R.m.s. deviation bond lengths (Å) | 0.004 | 0.005 |
| R.m.s. deviation bond angles (°) | 0.609 | 0.705 |
| Mean temperature factor (Å\(^2\)) | 27.65 | 29.51 |

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FIGURE 4. AMP binding site of PhPFK. A, stereo view of AMP-bound PhPFK nucleotide binding site. B, comparison of the nucleotide binding site of apo-PhPFK (green) and AMP-bound (cyan) PhPFK. These figures were generated in PyMOL.
ity in ADP-GKs and ADP-PFKs. ADP-PFKs are able to use GDP, to some extent, whereas ADP-GKs cannot. We made a S183E mutation and tested its ability to use different phospho-
yl donor compared with the wild type enzyme (Fig. 3A). There
was no difference in activity between the mutant and wild type
using ADP as a phosphoryl donor. However, the S183E mutant
displayed significantly less activity when the phosphoryl donor
was replaced with IDP, UDP, GDP, or CDP, compared with the
wild type.

One of the most intriguing features of these enzymes is the
position in that they can bind ADP in a way that both α- and β-
phosphates are recognized mainly in the same way as the
β- and γ-phosphates in the ATP-dependent enzymes. In this
respect, it has been proposed that the presence of a bulky
side chain in the bottom of the nucleotide binding crevice (Tyr-357 in the case of T. litoralis ADP-GK), below the sugar
part of the molecule, could account for the change in specific-
ity (20). Moreover, it was proposed that because ADP-
PFKs have leucine or isoleucine in this position they can
marginally use ATP as phosphoryl donor as it was seen for the
enzyme from P. furiosus (7). Because the corresponding
lateral chain of PfGK (Leu-340) is placed in the correspond-
ning position we tested directly the ability of this enzyme
to use ATP using the pyruvate kinase/lactate dehydrogen-
ase-coupled assay, which specifically consumes the ADP
produced. Surprisingly, no activity was detected with this
nucleotide.

Almost all phosphate transferring enzymes have Mg$^{2+}$ in
their active site thereby assisting with the phosphate transfer
through interactions with the β- and γ-phosphate groups of
ATP or the β-phosphate of ADP, when ADP is the phosphoryl
group donor. However, Mg$^{2+}$ was not observed in the PhPFK
structure or any of the ADP-GK structures. Although, 17 water
molecules were trapped in the AMP- and glucose-bound PjGK
structure, which offers sufficient space for both a magnesium
ion and the β-phosphate of ADP. Despite the prerequisite for
Mg$^{2+}$, this cation has never been observed in the structure of a
member of this superfamily, including PhPFK.

Reaction Mechanism—A conserved aspartate residue in
ribokinases, adenosine kinases, ADP-GKs, ADP-PFKs, and
ADP-GK/P FK is believed to be the catalytic base involved in the
phosphotransfer reaction of these kinases (Fig. 1). Mutations
of this residue in TIGK resulted in a significant reduction of glu-
cokinase activity (20). Similarly, mutation of the corresponding
aspartate in PhPFK (Asp-433) to alanine resulted in a loss of
PFK activity (Table 1).

Furthermore, a conserved argi-
nine residue that approaches the active site during the domain clos-
ing event associated with sugar binding is believed to attract the ter-
minal phosphate group of the phos-
phoryl donor, stimulating cleavage
of the phosphodiester bond and transfer of the phosphate group.
Mutation of Arg-205 in TlGK to ala-
nine resulted in <0.1% the activity of the wild type enzyme (20). We
observed the same effect upon mutation of the corresponding
arginine residue in PhPFK (Arg-185) to alanine (Table 1). These
two findings support a conserved mechanism among ADP-
GKs, ADP-PFKs, and ADP-GK/P FKs.

Substrate-induced Fit—A large conformational change
occurs in ADP-GKs as well as ATP-dependent hexokinases/
glucokinases and ribokinases upon binding their respective
substrates (Fig. 5) (20, 32–35). The flexible loops located
between the large and small domains form a hinge that folds,
closing the cleft between the two domains upon substrate bind-
ing (20, 32–35). This conformational change appears to be
essential for positioning the catalytic residues of ADP-GKs and
ribokinases (20, 34). The three ADP-GKs structures all display
varying amounts of domain closure (Fig. 5) (19–21). The apo-
PhGK is the most open conformation (Fig. 5) (21). There is a
rotation of about 20° between the large and small domain in the
ADP-bound TlGK structure compared with the PhGK
structure and an additional 20° rotation between the two domains
in the AMP- and glucose-bound PjGK structure compared with the
TlGK structure (Fig. 5) (19–21). It was initially reported that the
TlGK structure was the most open conformation because they were able to successfully soak ADP out of the
crystal, which indicates the absence of a large conformational
change (19). The PhGK structure clearly presented a more open
conformation, which led to the suggestion that TlGK had been
trapped in the ADP-bound conformation following the removal of the ADP from the crystal (21). However, ADP could
not be soaked into the PhGK crystal, which is thought to be due to
an ADP-dependent domain closing (21). Interestingly, no
such conformational change was observed between the apo-
and AMP-bound PhPFK structures. It could be that the β-phos-
phate of ADP is required to fully induce this conformational
change. The amine nitrogen of Arg-205 from TlGK is 3.8 Å
away from the β-phosphate of ADP and may form important
interactions that help to stabilize a more closed conformation
(19). One other possibility is that for PhPFK both co-factor and
substrate are required for the conformational change observed
as seen in other structures. In any case, the lack of conforma-
tional change in PhPFK in the presence of AMP is somewhat
surprising given the structure is from co-crystallization instead of
soaking.

Sugar-binding Site—Attempts to co-crystallize or soak crys-
tals with Fru-6-P or fructose-1,6-bisphosphate both in the pres-
ence or absence of ADP or nonhydrolyzable ADP analogues
were unsuccessful. Due to the overall similarity between the
PhPFK structures and ADP-GKs structures, we modeled
PhPFK in the closed conformation bound to Frut-6-P and AMP based on the AMP- and glucose-bound PfGK structure (Fig. 6). The primary sequence of PhPFK was threaded onto the PfGK structure bound to both AMP and glucose, and the resulting model was energy minimized. Frut-6-P was docked with the structure using AutoDock (36). All of the 10 lowest energy docking results placed the phosphate in the same position with only slight differences in the placement of the sugar moiety.

To validate our model and gain further insights into substrate specificity, relevant residues from the Frut-6-P binding pocket of PhPFK, the highly conserved residues Asn-15, Asp-17, Gln-97, Lys-158, Asn-160, Arg-185, Ser-189, Arg-191, Gln-224, Ser-263, and Asp-443 were mutated to alanine to establish their importance to substrate binding and catalysis. Of these, N15A, D17A, K158A, N160A, R185A, and R191A show the strongest differences in the placement of the sugar moiety.

FIGURE 6. Model of PhPFK bound to Frut-6-P and AMP. Docking model of the PhPFK-Frut-6-P complex. Where different, the positions of residues conserved in ADP-GKs are shown in orange. The red residue corresponds to the glutamate residue conserved in the bifunctional ADP-GK/PFK (Glu-82) and in ADP-GKs (Glu-88). Black dotted lines represent hydrogen bonding.

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To validate our model and gain further insights into substrate specificity, relevant residues from the Frut-6-P binding pocket of PhPFK, the highly conserved residues Asn-15, Asp-17, Gln-97, Lys-158, Asn-160, Arg-185, Ser-189, Arg-191, Gln-224, Ser-263, and Asp-443 were mutated to alanine to establish their importance to substrate binding and catalysis. Of these, N15A, D17A, K158A, N160A, R185A, and R191A show the most significant increase in their Km value for Frut-6-P (Table 1). Asn-160, Asn-15, and Arg-191 form one, two, and three hydrogen bonds with the phosphate group of Frut-6-P, respectively, as judged by the docked conformation (Fig. 6). Arg-185 and Asp-17 form hydrogen bonds with the sugar moiety of Frut-6-P (Fig. 6). As a result, loss of these interactions weakens PhPFK binding to its substrate. Lys-158 is too far to hydrogen bond with the phosphate of Frut-6-P in our model (Fig. 6). However, it is strictly conserved in PFKs and mutation of this residue has the most significant effect on Frut-6-P binding after the D17A mutation (Table 1). There are several possible explanations for this observation; however, it is most likely a result of the threading procedure itself, because there is no negative charge to stabilize the positive lateral chain of Lys-158 inside the binding pocket and therefore repelling it in the opposite direction. In fact, simple rotation of the side chain of Lys-158 in our model would result in a distance of less than 3.5 Å to the phosphate group of Frut-6-P. This was confirmed using a molecular dynamics simulation (see supplemental materials).

In this conformation, the lateral chains of Lys-158 and Arg-191 could form a salt bridge with the phosphate group of Frut-6-P, which would explain the behavior of the mutant enzyme.

Interestingly, the half of the sugar ring opposite to the endocyclic oxygen is recognized mainly in the same way as the comparable part of glucose by ADP-GKs. As expected the residues responsible of this like Asn-15, Asp-17, and Gly-96 are conserved in both specificities. As can be seen below, the sugar discrimination comes mainly by the interactions with the other half of the ring. This suggests that the sugar-binding site in the family is strongly plastic to accommodate a furanose-P and a pyranose with mainly the same residues, but still very selective to precisely discriminate between the two of them. Q224A and S263A contribute little to the sugar binding: both mutations result in small increases in Km for Frut-6-P of about 2–3-fold (Table 1). Surprisingly, Q97A, S189A, and D433A mutations lead to a small decrease in Km for Frut-6-P despite the loss of a hydrogen bond in the case of Q97A and D433A (Table 1). Also, Arg-185 and Asp-433 make contact with the C1 hydroxyl group, which supports the proposed catalytic mechanism. However, due to the lack of the phosphate group from ADP, the position of Arg-185 could be somewhat distorted. Nevertheless, our model is well supported by the mutational data.

Differences between ADP-PFKs and ADP-GKs—The conserved residues Asn-13, Asn-15, Asp-17, Gly-96, Gln-97 and Arg-185 form hydrogen bonds with Frut-6-P, as shown in Fig. 6A. Lys-158, Asn-160, and Arg-191 also form hydrogen bonds with Frut-6-P (Fig. 6). These residues are present in the bifunctional ADP-GK/PFK from M. jannaschii but the equivalent residues in ADP-GKs are non-conserved small residues, histidine and aspartate, respectively. A similar scenario was seen in a previous in silico study using the bifunctional enzyme from M. jannaschii (37). The positive charge on Arg-191 of PhPFK attracts the phosphate group of Frut-6-P, and is still compatible with glucose binding. However, when replaced by aspartate in ADP-GKs, the negatively charged side chain likely repels the phosphate group of Frut-6-P, which prevents ADP-GKs from binding Frut-6-P. To assess the role of Arg-191 in Frut-6-P binding, we made two mutations, R191A and R191E. The R191A mutation removes three hydrogen bonds to the phosphate group of Frut-6-P and causes a ~17-fold increase in the Km value for Frut-6-P (Table 1). However, as predicted, the charge reversal of the R191E mutation has a much more pronounced effect on Frut-6-P binding, a ~325-fold increase in Km (Table 1). Even so, the R191E mutant enzyme is not able to phosphorylate glucose, suggesting that the presence of this charged residue in the bottom of the sugar-binding site in all ADP-GKs contribute to sugar discrimination by hindering the protein from binding Frut-6-P and not by increasing the affinity for glucose.

The key difference between kinases that are capable of phosphorylating glucose and those that cannot is a conserved glutamate residue found in ADP-GKs (Glu-88 from PfGK) and ADP-GK/PFK (Glu-82) that is replaced with alanine in PhPFK (Ala-71). In the PfGK-glucose-AMP complex structure, the Glu-88 lateral chain forms a hydrogen bond with the C2 hydroxyl group of the bound glucose molecule. This interaction is thought to be critical for orienting the glucose molecule in the active site and it has also been highlighted as crucial for the sugar specificity in the past (6, 13, 37). As a result, ADP-PFKs cannot phosphorylate glucose. We tested this hypothesis by mutating Ala-71 to glutamate. As expected, this mutation has little effect on Frut-6-P binding compared with the wild type (Table 1). However, the A71E mutant enzyme can also bind glucose (Km = 3.95 ± 0.2 mM) and is capable of phosphorylating glucose (kcat of 2.68 ± 0.05 s−1) (Table 1). In contrast, the wild type enzyme displays no activity for glucose. This result demonstrates that Ala-71 is a key player in determining substrate specificity. Interestingly, the catalytic mechanism

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described before for the \( P_hPFK \) seems to be even more general because comparison of these features with the non-homologous ATP-dependent PFK (Pfk-1) from \( E. \) coli pointed out striking similarities.

In terms of catalysis, in Pfk-1, Asp-127 plays a critical role as a general base, increasing the nucleophilicity of the 1-hydroxyl of Fru-6-P by abstracting its proton and permitting attack on the \( \gamma \)-phosphate of the substrate ATP in a similar way as Asp-433 in \( P_hPFK \) (38). In addition, Arg-162 and Arg-243 interact with the 6-phosphate of Fru-6-P. Truncation of these residues to serine results in enzymes with decreased Fru-6-P binding ability and reduced cooperativity, but little change in catalytic ability in a very similar way to Lys-158 and Arg-191 (39). Then, it would appear that some structural features related to catalysis in ADP-dependent PFK have analogous counterparts in the ATP PFKs.

**Conclusion**—In summary, we determined the first crystal structure of an ADP-dependent phosphofructokinase both alone and in complex with AMP and through comprehensive mutagenesis we identified residues that were critical for both the phosphotransfer reaction and substrate binding. Our results demonstrate that the overall structure of ADP-PFK is similar to that of ADP-GK and that the two share a common mechanism of action. However, unlike the available ADP-GK structures, no conformational change was observed upon nucleotide binding. This was unexpected and may indicate that the nucleotide-dependent conformational change is a non-essential part of the ADP-dependent sugar kinase mechanism. Moreover, we identified four key residues responsible for the sugar binding specificity. Using this knowledge, we were able to generate a mutant \( P_hPFK \) that is capable of phosphorylating both Fru-6-P and glucose.

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