FUNCTIONAL LINKAGE OF ADENINE NUCLEOTIDE BINDING SITES IN THE MAMMALIAN MUSCLE 6-PHOSPHOFRUCTOKINASE

Antje Brüser¹, Jürgen Kirchberger¹, Marco Kloos², Norbert Sträter², Torsten Schöneberg¹*

From the ¹Institute of Biochemistry, Molecular Biochemistry, Medical Faculty, University of Leipzig, Johannisallee 30, 04103 Leipzig, Germany, ²Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine, University of Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany

Running Title: Regulation of mammalian 6-phosphofructokinase

*Address correspondence to: Torsten Schöneberg, Institut für Biochemie, Abt. Molekulare Biochemie, Medizinische Fakultät, Universität Leipzig, Johannisallee 30, 04103 Leipzig, Germany; Tel. +49-341-9722150; Fax +49-341-9722159; e-mail: schoberg@medizin.uni-leipzig.de

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Background: Crystal structures of 6-phosphofructokinases revealed nucleotide binding sites with unknown functional relevance.

Results: Function of two allosteric nucleotide binding sites was determined by mutagenesis and kinetic studies and revealed reciprocal linkage of both.

Conclusion: Activity of mammalian Pfk is regulated by structurally linked new allosteric sites.

Significance: Reciprocal linkage between allosteric binding sites evolved convergent in prokaryotes and eukaryotes.

SUMMARY

6-Phosphofructokinases (Pfk) are homo- and heterooligomeric, allosteric enzymes that catalyze one of the rate-limiting steps of the glycolysis: the phosphorylation of fructose 6-phosphate at position 1. Pfk activity is modulated by a number of regulators including adenine nucleotides. Recent crystal structures from eukaryotic Pfk revealed several adenine nucleotide binding sites. Herein, we determined the functional relevance of two adenine nucleotide binding sites through site-directed mutagenesis and enzyme kinetic studies. Subsequent characterization of Pfk mutants allowed the identification of the activating (AMP, ADP) and inhibitory (ATP, ADP) allosteric binding sites. Mutation of one binding site reciprocally influenced the allosteric regulation through nucleotides interacting with the other binding site. Such reciprocal linkage between the activating and inhibitory binding sites is in agreement with current models of allosteric enzyme regulation. Since the allosteric nucleotide binding sites in eukaryotic Pfk did not evolve from prokaryotic ancestors, reciprocal linkage of functionally opposed allosteric binding sites must have developed independently in prokaryotic and eukaryotic Pfk (convergent evolution).

The ATP-dependent 6-phosphofructokinase (EC 2.7.1.11, phosphofructokinase-1, ATP: D-fructose-6-phosphate-1-phosphotransferase; Pfk) catalyzes the phosphorylation of fructose 6-phosphate (Frut-6-P) to fructose 1,6-bisphosphate (Frut-1,6-P₂). This irreversible reaction is considered to be one of the rate-limiting steps of glycolysis (1-3). In eukaryotes the Pfk activity is modulated by a number of allosteric regulators, e.g. ATP, AMP, NH₄⁺, fructose 2,6-bisphosphate (Frut-2,6-P₂), citrate and acyl-CoA (4). Most eukaryotic Pfk are homo- and heterooligomeric enzymes consisting of subunits which evolved from a single ancestor prokaryotic Pfk through gene duplication and mutational events (5,6).

In eukaryotes the N-terminal half of a Pfk subunit mediates the catalytic function, whereas in the C-terminal half allosteric ligand binding sites have evolved from former catalytic and regulatory sites (5,7,8). It is assumed that specific amino acid residues involved in...
catalytic and regulatory functions of PfK from *E. coli* (9,10) are conserved in yeast and mammalian PfK genes. For example, citrate inhibits the enzyme through interaction with sites that have evolved from a duplicated allosteric site (PEP binding site) (11,12) whereas sites for activators, e.g. Fru-2,6-P₁, have evolved from the catalytic site (Fru-6-P) of the ancestral precursor (13,14). In contrast, the ATP substrate binding site from the ancestral prokaryotic PfK did not evolve to a new inhibitory ATP binding pocket (13,15) and it remains unclear as to how adenine nucleotides implement their allosteric inhibitory (ATP) and activating (AMP) effects. Note that ATP serves as a substrate and as an allosteric inhibitor of eukaryotic PfK. ADP can act as an activator at µM concentrations but inhibits eukaryotic PfK activity at mM concentrations (16).

Until recently, no high resolution structure of eukaryotic PfK was available to prove the conservation and the functional relevance of allosteric ligand binding sites.

In 2011, crystal structures of three eukaryotic PfK from the yeasts *Pichia pastoris* (15) and *S. cerevisiae* (13) and from rabbit muscle (13) were determined. In the crystal structure of *P. pastoris* PfK an additional ATP binding pocket was found in each β chain which was presumed to be an inactivating site (15). In the structure of rabbit muscle PfK this ATP binding site was also discovered in the N-terminal domain of each subunit with either ADP or ATP bound to it. An additional novel ATP binding site was found in the center of each subunit (13). This was proposed as an activating (ADP binding) site because replacement by ATP failed (Fig. 1). Neither of these two binding sites evolved from a regulatory or catalytic binding site in bacterial PfK. Fig. 1B shows a superposition of the *E. coli* PfK with its two equivalent effector binding sites at the subunit interfaces. The superposition of rabbit and *E. coli* PfK suggests that the effector binding sites of the bacterial protein are maintained in the mammalian enzyme but only a phosphate ion is bound to this site. Since ADP is found in at least two binding sites the functional relevance of the individual binding sites is still unclear. Furthermore, it is unknown whether additional binding sites exist in PfK mediating the activating and inhibitory effects of adenine nucleotides. There is no crystal structure with AMP bound to PfK available yet.

Here, we experimentally addressed the functional relevance of the canonic activating and inhibitory adenine nucleotide binding sites through mutagenesis and kinetic studies. We functionally qualified the adenine nucleotide binding sites and identified a reciprocal linkage between these allosteric binding sites.

**EXPERIMENTAL PROCEDURES**

**Materials**

If not otherwise stated, all chemicals and standard substances were purchased from Sigma Aldrich (Taufkirchen, Germany), C. Roth GmbH (Karlsruhe, Germany), Roche Applied Science (Mannheim, Germany), Fermentas GmbH (St. Leon-Rot, Germany) and BD (New Jersey, USA). Restriction enzymes and primers were purchased from New England Biolabs (Frankfurt/Main, Germany) and Invitrogen (Karlsruhe, Germany), respectively.

**Methods**

**Strains and growth conditions** - The *E. coli* strain DH5α was used for the cloning experiments. Transformants were selected on LB medium containing 100 µg/ml ampicillin. The yeast strain for expressing human wild type and mutant muscle PfK was *Saccharomyces cerevisiae* HD114-8D (MATα Scpfk1::HIS3 Scpfk2::HIS3 his3-11,15 leu2-3,112 ura3-52) carrying deletion in both yeast PfK genes (17,18). Preparation of competent cells was performed as described (17). Selection for transformation of wild type and mutant PfK was performed at 30°C in YP medium (1 % yeast extract, 2 % bacto peptone) containing 2 % glucose as a carbon source.

**Selection of target residues for mutagenic analysis** – Asn³⁴¹, Ser⁷⁷, Lys⁶⁷⁸, Val²²⁸, His²⁴², Arg⁵⁴⁶ and Lys³⁸⁶ were selected based on the crystal structure of rabbit skeletal muscle PfK (PDB: 3O8N, 3O8L). These residues participate in the proposed ADP activator and the ATP inhibitor binding sites (13,15) and are conserved between the human and rabbit muscle PfK.

**Construction of mutant PfK** – All mutants were constructed using PCR-based site-directed mutagenesis and the fragments were cloned into the plasmid pJH71PFK (kindly provided by Prof. Dr. Jürgen J. Heinisch, University of Osnabrück (18)). The resulting plasmids were sequenced to ensure sequence correctness.

**Expression and purification of recombinant enzymes** – Transformation of yeast strain HD114-8D was performed with LiAc/ssDNA/PEG (19). Transformants were grown in liquid medium with shaking at 30°C. After 24 h the cells were harvested by centrifugation at 5700 x g for 15 min at 4°C and washed twice with water and once with a 50 mM potassium phosphate buffer, pH 7.2, containing

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1 mM EDTA, 5 mM ME and 0.5 mM PMSF (buffer A). Cell disruption was performed according to (20) in buffer A with 50 mM NaF, 50 µM Fru-1,6-P_2 and protease inhibitor cocktail (Roche Applied Science). Following steps were carried out at 4°C unless described otherwise. The mixture was centrifuged at 75 000 x g for 30 min. The supernatant was subjected to protamine sulphate precipitation (0.2 % w/v) (21) with stirring for 5 min. After centrifugation the pellet was discarded and the supernatant was precipitated with PEG6000 (5 % w/v) for 30 min with stirring. After centrifugation as before, the pellet was dissolved in buffer B (50 mM Tris/HCl, pH 8.0, 50 µM Fru-1,6-P_2, 0.1 mM EDTA, 5 mM ME, 50 mM NaF, 0.5 mM PMSF; according to (21)). Cibacron Blue F3GA-Sephadex™ G75 (prepared according to (22)), equilibrated with buffer B, was added to the solution and stirred for 30 min (binding capacity: 15 units/g gel wet weight). The gel was washed on a funnel with buffer B. Elution was done with buffer A, pH 8.0, containing 2 mM Fru-6-P and 5 mM ATP. The enzyme was concentrated by ultrafiltration (Omega 100 kDa-membrane, Pall Life Sciences, Dreieich, Germany). Glycerol was added to a final concentration of 20 % (v/v) and the solution can be stored at -20 °C for weeks without loss of activity. For ion exchange chromatography the concentrated enzyme sample (without glycerol) was diluted with 0.8 vol. 0.5 x buffer A, pH 8.0 and loaded on a Resource Q-Column (6 ml, GE Healthcare, Munich, Germany), equilibrated with buffer A, pH 8.0. After washing with buffer A, pH 8.0, the enzyme was eluted with a linear gradient of KCl (0-200 mM) in buffer A, pH 8.0. Fraction of Pfk activity were pooled, supplemented with 2 mM ATP and 50 µM Fru-1,6-P_2. After concentration by ultrafiltration (Pall Life Sciences, Omega 100 kDa-membrane, Pall Life Sciences, Dreieich, Germany) and Vivaspin 6 (Sartorius, Göttingen, Germany) 20 % glycerol (v/v) was added and the mixture stored at -20°C for weeks without loss of activity. For ion exchange chromatography the concentrated enzyme sample (without glycerol) was diluted with 0.8 vol. 0.5 x buffer A, pH 8.0 and loaded on a Resource Q-Column (6 ml, GE Healthcare, Munich, Germany), equilibrated with buffer A, pH 8.0. After washing with buffer A, pH 8.0, the enzyme was eluted with a linear gradient of KCl (0-200 mM) in buffer A, pH 8.0. Fraction of Pfk activity were pooled, supplemented with 2 mM ATP and 50 µM Fru-1,6-P_2. After concentration by ultrafiltration (Pall Life Sciences, Omega 100 kDa-membrane, Pall Life Sciences, Dreieich, Germany) 20 % glycerol (v/v) was added and the mixture stored at -20°C. Gel permeation chromatography was done on a BioSep SEC-S4000 (600 x 21.2 mm; Phenomenex, Aschaffenburg, Germany). The column was equilibrated with buffer A containing 50 µM Fru-1,6-P_2, pH 7.5. Fractions with Pfk activity were pooled, 2 mM ATP added and the enzyme solution was concentrated as before. The purified enzyme can be stored with 30 % glycerol (v/v) at -20°C for two weeks without loss of activity. For kinetic studies of wild type and mutant Pfk the partially purified enzyme after affinity chromatography with F3GA-Sephadex™ was used.

Pfk activity assay - During preparation, Pfk activity was measured spectrophotometrically at 340 nm and 25°C according to (20) except pH was 8.5 and 1.2 mM ATP was used. The reaction was started with the addition of a 2-5 µl enzyme sample.

Kinetic studies were performed in 50 mM HEPES, 100 mM KCl, 5 mM MgCl_2, pH 7.0, 0.2 mM NADH, 0.45 U/ml aldolase, 4.5 U/ml triosephosphate isomerase and 1.5 U/ml glycerol phosphate dehydrogenase. ATP, Fru-6-P, AMP and ADP were used as indicated. In the case of the ATP-inhibition experiments the MgCl_2 concentration was 15 mM. Auxiliary enzymes were dialyzed before use (Micro Bio-Spin 6, Bio-Rad Laboratories, Munich, Germany). The reaction was started by the addition of 5 µl enzyme sample appropriately diluted with 50 mM sodium phosphate buffer, pH 7.2, containing 10 % glycerol. Curve fittings for kinetic parameters were generated by either Michaelis-Menten or Hill equations using Prism (GraphPad Software, Inc. La Jolla). Thus, the hyperbolic and the sigmoid parts of the curves (V_min to V_max) were fit to Michaelis-Menten and Hill equations, respectively. To exclude artifacts in the determination of the kinetic parameters using partially purified enzymes, wild type and two mutants (N^341A, R^246A/K^386A) were purified to homogeneity. Since there were no differences in the kinetic parameters, all mutant Pfk were partially purified (see above) and tested in kinetic assays.

RESULTS

Identification of the activating site

In the crystal structure of rabbit skeletal muscle Pfk, the diphosphate moiety of ADP in the allosteric activation site is mainly coordinated by Ser^377 and Lys^678 (Fig. 1C). Mutation of the latter two residues to Ala significantly increased K_0.5 for ADP (Table 1). The K_0.5 for ADP of the double mutant S^377A/K^678A was further increased (see Table 1) indicating that this ADP binding site is indeed the allosteric activating site in Pfk. The AMP activation of S^377A and K^678A was less pronounced (see Table 1) probably because of weaker or absent interaction between these residues and the monophosphate moiety of AMP.

Next, we mutated Asn^341 to Ala, which interacts with the ribose of ADP (Fig. 1C) and probably also AMP. In contrast to S^377A and K^678A, N^341A not only showed increased K_0.5 values for ADP but also significantly reduced enzyme activation by AMP (Fig. 2A/B, Table 1).
ADP and ATP reduced Pfk activity at mM concentrations (Fig. 2A/C) most likely via binding to the inhibitory nucleotide binding site (Fig. 1D/E). Strikingly, $K_i$ values of ADP and ATP were significantly reduced in N341A and S377A/K678A (Fig. 2A/C, Table 1). This effect was found even in the presence of effectors (Fig. 2C, Table 1; data for ADP not shown). In contrast, $K_i$ values of ADP and ATP remained unchanged in S377A and K678A.

To test whether mutations in the allosteric activator site influence the substrate dependency of the catalytic activity, the influence of Fru-6-P concentrations was tested in the presence of different effectors. As shown in Fig. 3 and Table 2, activities of wild type and mutant enzymes exhibited cooperativity to Fru-6-P without effectors. In the presence of the effectors AMP and ADP the affinity to this substrate was increased. However, specifically N341A and S377A/K678A displayed significantly increased $S_{0.5}$ values in comparison to the wild type under these conditions.

**Identification of the inhibitory site**

ADP and ATP inhibit Pfk activity by binding to one or more allosteric inhibitory sites. In the rabbit muscle Pfk crystal structure, ADP and ATP in the potential allosteric inhibition site are mainly coordinated by Val228, His242, Arg246, and Lys386 (Fig. 1D/E). V228A had no significant effects on Pfk activity compared to the wild-type enzyme (data not shown) most probably because of the very conservative structural change. In contrast, mutation of the other three residues to Ala significantly increased $K_i$ for ATP and ADP (Fig. 4, Table 3). $K_i$ values for ATP and ADP of the double mutant R246A/K386A were further increased (Fig. 4, Table 3), indicating that this nucleotide binding site is indeed the allosteric inhibition site of ADP and ATP in mammalian Pfk. Interestingly, all mutants showed significantly higher basal activity in the absence of the activators AMP and ADP (Fig. 4B, Table 3), suggesting that the enzyme is constitutively activated due to mutation in the allosteric inhibitor site. The Fru-6-P dependency of the Ala mutants of His242, Arg246, and Lys386 presented a Michaelis-Menten kinetic indicating a lack of cooperativity (data not shown).

Finally, we combined mutations of the allosteric activating (N341A) and the inhibitory (R246A/K386A) sites in the triple mutant N341A/R246A/K386A. This mutant was also constitutively activated but with a lower extent (66 % of maximum activity). The $K_i$ values for ATP and ADP compared to mutants of the inhibitory allosteric site alone were significantly decreased (Table 3, Fig. 4).

**DISCUSSION**

Pfk activity underlay strong allosteric regulation by several effectors including adenine nucleotides. Crystal structures of prokaryotic and eukaryotic Pfk revealed a number of adenine nucleotide molecules bound in the catalytic center as well as in allosteric binding sites (Fig. 1A/B). In the crystal structure of rabbit skeletal muscle Pfk (13) ADP was found in two binding sites (Fig. 1C/D) which were different to the well characterized allosteric binding sites in prokaryotic Pfk (Fig. 1B). Because ADP can activate (at µM concentration) and also inactivate (at mM concentration) the enzyme, we approached the functional relevance of these two nucleotide binding sites by site-directed mutagenesis. In one crystal structure of the rabbit skeletal muscle, Pfk ATP was found in one of the two ADP binding sites (Fig. 1E) suggesting this as the inhibitory allosteric site, however an experimental proof was missing.

The single mutations of binding relevant residues in the potential activating binding site (N341A, S377A, K678A) shifted the $K_{0.5}$ values of ADP to higher concentrations (Table 1). Increased $K_{0.5}$ values of AMP were found for S377A/K678A and N341A (Table 1), strongly supporting annotation of this nucleotide binding site as the allosteric activating binding site for AMP and ADP. Interestingly, the $K_{0.5}$ values for AMP were unchanged in the single mutants (S377A, K678A). This suggests that, in contrast to ADP, where the diphosphate moiety of ADP is coordinated by Ser377 and Lys678 (Fig. 1C), binding of the monophosphate residue of AMP may involve additional or other residues. Only a crystal structure of muscle Pfk with AMP bound may clarify detailed coordination of this nucleotide in the activating allosteric site.

Mutation of Asn341 was more efficient in increasing the $K_{0.5}$ values of both ADP and AMP (Table 1). Since the backbone nitrogen atom of N341 interacts with the ribose of ADP, the side chain is probably involved in the transmission of the allosteric signal or necessary for the correct positioning of the backbone chain. Most strikingly however, $K_i$ values of ADP and ATP were significantly shifted towards lower concentrations suggesting an increase of the effector affinity at the allosteric inhibitory binding site or an enhanced transduction of the inhibitory effect of ATP and ADP (Fig. 2A/C).
Single Ala mutations of His242, Arg246, and Lys386 and double mutation (R246A/K386A) in the putative inhibitory binding site reduced the inhibitory potency of ATP and ADP (Table 3), strongly supporting the postulated function as the inhibitory allosteric site. However, PfK activity was almost at the maximum in the absence of the activator AMP or ADP (Fig. 4B) indicating constitutively activated mutant enzymes. The maximum activity of e.g. R246A/K386A was not significantly different from the wild type and the specific activity of highly purified Ala mutants did not differ from the wild type enzyme under optimal conditions excluding gross structural changes in the mutants. It appears that mutations of the inhibitory allosteric site “locked” the enzyme in an active conformation mimicking the state in the presence of AMP. This is supported by the fact that Fru-6-P dependency of the Ala mutants (H242A, R246A, K386A) presented a Michaelis-Menten kinetic indicating a lack of cooperativity. However, additional mutation on the activating allosteric site (N341A/R246A/K386A) did not reverse this constitutively activated conformation but improved ATP inhibition compared to R246A/K386A (see Fig 4A/B). This indicates a more complex interplay of the allosteric sites or several (dissociable) active states.

From recent studies we know that the smallest active enzyme form of mammalian PfK consists of four subunits but higher oligomeric complexes exists, which are also functional (23,24). Physiologically, AMP and µM concentration of ADP promote enzyme activation and mM concentrations of ADP and ATP promote enzyme inhibition. This allosteric regulation directly couples cellular energy metabolism to cellular ATP/AMP ratio. Our data now suggests that this ATP/AMP-driven allostery is further fine-tuned by a reciprocal linkage of the two functionally opposed allosteric sites. Mutation of the activating allosteric site reduces the activating effect of AMP and ADP and synergistically increases the inhibitory effect of ATP and ADP. Vice versa, mutation of the inhibitory allosteric site increases $K_i$ values of ATP and ADP and results in a constitutively activated enzyme. One may speculate that mutation or ligand occupation of one allosteric site lead to structural changes followed by changes in the affinity of the opposite allosteric site. For example, occupation of the allosteric activator site by AMP in the wild type PfK decreases the affinity of ATP at the allosteric inhibitory site. Indeed, early studies with the skeletal muscle PfK showed that the binding of AMP resulted in an antagonism of ATP inhibition by decreasing the affinity of ATP binding (25). Further evidence for such mechanistic explanation comes from the crystal structures of the prokaryotic PfK from E. coli. In the inactive state, the AMP binding site is structurally different compared to the active state (10). Crystal structure of eukaryotic PfK clearly showed that the allosteric nucleotide binding sites are different from those in the prokaryotic PfK (13,15). Therefore, reciprocal linkage of functionally opposed allosteric binding sites must have developed independently in prokaryotic and eukaryotic PfK giving an impressive example of convergent evolution at the molecular level.

Although a reciprocal linkage of functionally opposite allosteric binding sites is in agreement with the extended Monod-Wyman-Changeux model of allostery (26) this allostry model has been shown inappropriate in a certain number of cases. For prokaryotic PfK, the classical two-state model is not sufficient to describe the allosteric behavior (27) so additional models of allostery were developed (26-29). Since mammalian PfK are even more complex (in terms of structure and regulation) than prokaryotic PfK, appropriate crystal structures of the various states are required to clarify whether the functionally opposed allosteric sites are linked within a subunit or between subunits.

In sum, we assigned two new allosteric adenine nucleotide binding sites to be the activating and inhibitory nucleotide binding sites. The latter is occupied by ATP or ADP. The novel ADP binding site found in the crystal structure of mammalian PfK is the activating allosteric site and binds also AMP. Our data strongly supports that activating and inhibitory allosteric binding sites do not only regulate the catalytic PfK activity but also modulate the properties of each other in a reciprocal manner. This crosstalk between the allosteric nucleotide binding sites may further fine-tune PfK activity depending on the ATP/AMP ratio.

REFERENCES

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Abbreviations
Pfk, 6-phosphofructokinase; Fru-6-P, fructose 6-phosphate; Fru-2,6-P₂, fructose 2,6-bisphosphate, Fru-1,6-P₂, Fructose 1,6-bisphosphate; YP, yeast extract peptone; ME, 2-mercaptoethanol
FIGURE LEGENDS

Fig. 1. Adenine nucleotide binding sites in the crystal structure of the rabbit muscle Pfk. (A) Crystal structure of rabbit skeletal muscle Pfk (13). One monomer of the dimer found in the crystals is shown. The N-terminal half is colored in blue and the C-terminal half in yellow. Three different ADP binding sites were identified in the crystal structure: the catalytic center, putative inhibitory and activating allosteric sites. (B) Superposition of rabbit skeletal muscle Pfk (colored as in (A)) with E. coli Pfk (depicted as a transparent cartoon, PDB id 1PFK (30)). One monomer of the homotetrameric bacterial enzyme superimposes on the N-terminal half and one onto the C-terminal half. The two prokaryotic effector-binding sites of the dimer are occupied by ADP (brown). The bacterial effector-binding sites are located at the subunit interfaces, i.e. between the N- and C-terminal halves in the mammalian Pfk. (C) In the activating allosteric sites, the diphosphate moiety of ADP interacts via hydrogen bonds with Ser^{377} and Lys^{378} whereas the ribose contacts Asn^{341}. In the potential inhibitory allosteric site ADP (D) and ATP (E) are coordinated by Val^{228}, His^{242}, Arg^{246}, and Lys^{386}.

Fig. 2. Effect of ATP, ADP and AMP on the activity of wild type and mutant N^{341}A Pfk. The effect on the Pfk activity of increasing concentrations of ADP (A) and AMP (B) at the wild type and N^{341}A was determined with 0.5 mM Fru-6-P and 1 mM ATP. Activity is expressed relative to maximal activity (V) for each enzyme under these conditions (V values of wild type were 295 U/ml and 233 U/ml for AMP and ADP, respectively and 147 U/ml and 57 U/ml for N^{341}A). (C) For ATP, assays were performed at 2 mM Fru-6-P. Activity is expressed as a relation between the measured (v) and maximal possible (V) activity for each enzyme under these conditions (V values of wild type were 396 U/ml and 434 U/ml without effectors and 1 mM AMP, respectively and 147 U/ml and 247 U/ml for N^{341}A). Dotted line without effectors; continuous line with 1 mM AMP. Data are means ± SEM of three independent experiments each performed in duplicate.

Fig. 3. Fru-6-P dependency of wild type and mutant N^{341}A Pfk activity. Assays were performed (A) without effectors and (B) with 1 mM AMP (continuous line) and 0.82 mM ADP (dotted line) with fixed concentration of 1 mM ATP and varying concentrations of Fru-6-P as indicated. Activity is expressed relative to maximal activity (for wild type 383, 374 and 359 U/ml without effectors, 1 mM AMP and 0.82 mM ADP, respectively and 116, 254 and 126 U/ml for N^{341}A). Data are means ± SEM of three independent experiments each performed in duplicate.

Fig. 4. Effect of ATP and ADP on the activity of wild type Pfk and various mutations at the inhibitory allosteric site. The effect on the Pfk activity of increasing concentrations of (A) ATP at the wild type, R^{246}A, K^{386}A, R^{246}A/K^{386}A and the enzyme mutated additionally at the activating site (N^{341}A/R^{246}A/K^{386}A) was determined at 2 mM Fru-6-P. Activity is expressed relative to maximal activity for each enzyme under these conditions (V values were 274, 233, 213 and 164 U/ml for R^{246}A, K^{386}A, R^{246}A/K^{386}A, and N^{341}A/R^{246}A/K^{386}A, respectively). To compare data with N^{341}A see Fig. 2C (B) The activity of wild type and the mutants was measured at 0.5 mM Fru-6-P and 1 mM ATP in absence and presence of 4.5 mM ADP (inhibitory concentration). Under both conditions the wild type and N^{341}A displayed no significant activity. Activity is expressed relative to maximal activity (V) for each enzyme under these conditions (V values: wild type 295 U/ml, H^{242}A 270 U/ml, R^{246}A 237 U/ml, K^{386}A 171 U/ml, R^{246}A/K^{386}A 250 U/ml, N^{341}A/R^{246}A/K^{386}A 209 U/ml). Data are means ± SEM of three independent experiments each performed in duplicate.
TABLES

Table 1. Effect of AMP, ADP and ATP on kinetic properties of the wild type human muscle Pfk and Pfk mutated at the activating allosteric site.

Kinetic properties of wild type and mutant Pfk were determined. Thus, the hyperbolic and the sigmoid parts of the curves (V\textsubscript{min} to V\textsubscript{max}) were fit to Michaelis-Menten ($K_m$) and Hill ($K_{0.5}$, $K_i$) equations, respectively. For ATP, assays were performed with 2 mM Fru-6-P and either with 1 mM AMP or 0.82 mM ADP, respectively. The kinetic parameters for AMP and ADP were determined at 0.5 mM Fru-6-P and 1 mM ATP. Residual Pfk activity was defined as a percentage of enzyme activity at 2.3 mM ADP and maximal activity for each enzyme under these conditions. The values are means ± SEM of three independent experiments.

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<th>Parameter</th>
<th>Wild type</th>
<th>N\textsuperscript{341}A</th>
<th>S\textsuperscript{377}A</th>
<th>K\textsuperscript{678}A</th>
<th>S\textsuperscript{377}/K\textsuperscript{678}A</th>
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<td>$K_m$ (µM)</td>
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<td>52.8 ± 3.6</td>
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<td>$K_i$ (mM)</td>
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<td>5.2 ± 0.1</td>
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<td>$K_m$ (µM)</td>
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<td>$K_{0.5}$ (µM)</td>
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<td>610 ± 13.1</td>
<td>454 ± 13.0</td>
<td>422 ± 3.4</td>
<td>506 ± 14.1</td>
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<td>$K_i$ (mM)</td>
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<td>1.6 ± 0.1</td>
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<td>$K_{0.5}$ (µM)</td>
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<td>Residual activity (%)</td>
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</tbody>
</table>
Table 2. Saturation kinetics for Fru-6-P on the wild type human muscle Pfk and Pfk mutated at the activating allosteric site.

Assays were carried out at in the presence of 1 mM ATP and indicated concentrations of effectors. By fitting with the Hill equation the parameters were determined with $S_{0.5}$ as the substrate concentration at half-maximal activity and $n_H$ as hill coefficient. The values are means ± SEM of three independent experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>N$_{341}$A</th>
<th>S$_{377}$A</th>
<th>K$_{678}$A</th>
<th>S$<em>{377}$A/K$</em>{678}$A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{0.5}^{Fru-6-P}$ (mM)</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>$n_H^{Fru-6-P}$</td>
<td>2.6 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>1 mM AMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_{0.5}^{Fru-6-P}$ (mM)</td>
<td>0.09 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>$n_H^{Fru-6-P}$</td>
<td>1.4 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>0.82 mM ADP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_{0.5}^{Fru-6-P}$ (mM)</td>
<td>0.19 ± 0.01</td>
<td>0.61 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.42 ± 0.01</td>
<td>0.58 ± 0.01</td>
</tr>
<tr>
<td>$n_H^{Fru-6-P}$</td>
<td>2.7 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>2.5 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>
Table 3. Functional characterization of human muscle Pfk modified in the inhibitory allosteric site.

Kinetic parameters for ATP of wild type and mutant Pfk were determined at 2 mM Fru-6-P and indicated effector concentrations. Thus, the hyperbolic and the sigmoid parts of the curves (V_min to V_max) were fit to Michaelis-Menten (K_m) and Hill (K_{0.5}, K_i) equations, respectively. For comparing data of the triple mutant with N^{341}A see Table 1. For ADP, kinetic properties were determined with 0.5 mM Fru-6-P and 1 mM ATP. Residual Pfk activity was defined as percentage of enzyme activity at 4.5 mM ADP and maximal activity of each enzyme under these conditions. The values are means ± SEM of three independent experiments. nd: not detectable.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>H^{242}A</th>
<th>R^{246}A</th>
<th>K^{386}A</th>
<th>R^{246}A/K^{386}A</th>
<th>N^{341}A/R^{246}A/K^{386}A</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m^{ATP} (µM)</td>
<td>42.5 ± 2.0</td>
<td>38.2 ± 2.0</td>
<td>43.2 ± 1.3</td>
<td>52.4 ± 4.7</td>
<td>47.7 ± 2.4</td>
<td>35.6 ± 2.0</td>
</tr>
<tr>
<td>K_i^{ATP} (mM)</td>
<td>2.0 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>8.6 ± 0.3</td>
<td>5.6 ± 0.1</td>
<td>&gt; 10.0</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>K_m^{ATP} (µM)</td>
<td>49.8 ± 2.7</td>
<td>33.8 ± 2.0</td>
<td>50.8 ± 5.0</td>
<td>35.6 ± 1.9</td>
<td>42.5 ± 1.5</td>
<td>63.0 ± 2.3</td>
</tr>
<tr>
<td>K_i^{ATP} (mM)</td>
<td>8.5 ± 0.1</td>
<td>&gt; 10.0</td>
<td>&gt; 10.0</td>
<td>&gt; 10.0</td>
<td>&gt; 10.0</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>K_m^{ATP} (µM)</td>
<td>169 ± 9.9</td>
<td>114 ± 4.4</td>
<td>166 ± 7.1</td>
<td>168 ± 6.0</td>
<td>234 ± 13.6</td>
<td>220 ± 7.5</td>
</tr>
<tr>
<td>K_i^{ATP} (mM)</td>
<td>5.3 ± 0.1</td>
<td>&gt; 10.0</td>
<td>&gt; 10.0</td>
<td>9.5 ± 0.1</td>
<td>&gt; 10.0</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>K_{0.5}^{ADP} (µM)</td>
<td>83.6 ± 0.9</td>
<td>n.d.</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>K_i^{ADP} (mM)</td>
<td>2.1 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Residual activity at 4.5 mM ADP (%)</td>
<td>0</td>
<td>38.4 ± 1.2</td>
<td>41.7 ± 0.1</td>
<td>33.1 ± 0.1</td>
<td>33.2 ± 0.5</td>
<td>21.9 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 1

A

B

C

D

E
Figure 2

A

Relative activity (v/V) vs. ADP [mM]

B

Relative activity (v/V) vs. AMP [mM]

C

Relative activity (v/V) vs. ATP [mM]

Figure 3

A

Relative activity (v/V) vs. F6P [mM]

B

Relative activity (v/V) vs. Fru-6-P [mM]
Figure 4

A

B

Relative activity (v/V) vs. ATP [mM]

Relative ATPase activity

WT
R246A
N341A/R246A/K386A
K386A
R246A_K386A

ATP [mM]

0.2
0.4
0.6
0.8
1.0

0
0.2
0.4
0.6
0.8
1.0

0 mM ADP
4.5 mM ADP

WT
H242
K246
R246
A246
A/K246

0 mM ADP
4.5 mM ADP

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FUNCTIONAL LINKAGE OF ADENINE NUCLEOTIDE BINDING SITES IN THE MAMMALIAN MUSCLE 6-PHOSPHOFRUCTOKINASE
Antje Bruser, Jurgen Kirchberger, Marco Kloos, Norbert Strater and Torsten Schoneberg

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