Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structure of E69Q mutant of human muscle fructose-1,6-bisphosphatase

Marek Zarzycki,[a] Robert Kołodziejczyk,[b,c] Ewa Maciaszczyk-Dziubinska,[d] Robert Wysocki,[d] Mariusz Jaskolski[b,e] and Andrzej Dzugaj[a]*

[a]Institute of Genetics and Microbiology, Faculty of Biological Sciences, University of Wroclaw, ul. Przybyszewskiego 63/77, 51-148 Wroclaw, Poland, [b]Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Grunwaldzka 6, 60-780 Poznan, Poland, [c]Macromolecular X-ray Crystallography Group, Structural Biology and Biophysics, Institute of Biotechnology, University of Helsinki, Helsinki, Finland, [d]Institute of Plant Biology, Faculty of Biological Sciences, University of Wroclaw, Kanonia 6/8, 50-328 Wroclaw, Poland, and [e]Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Z. Noskowskiego 12/14, 61-704 Poznan, Poland

Correspondence e-mail: dzugajan@biol.uni.wroc.pl

Received 1 August 2011
Accepted 21 October 2011

PDB References: FBPase–AMP, 3ifa; FBPase–AMP–F6P, 3ifc.

Human fructose-1,6-bisphosphatase is an allosteric enzyme that is regulated by different ligands. There are only two known isozymes in human tissues: the liver isozyme (the key enzyme of gluconeogenesis), which is regulated by fructose 2,6-bisphosphate, and its muscle counterpart (participating in glycogen synthesis), which is regulated by calcium ions. AMP, which is an allosteric inhibitor of both isozymes, inhibits the muscle isozyme with an $I_{0.5}$ that is 35–100 times lower than for the liver isozyme and the reason for this difference remains obscure. In studies aiming at an explanation of the main differences in the regulation of the two isozymes, it has been shown that only one residue, in position 69, regulates the sensitivity towards calcium ions. As a consequence of this finding, an E69Q mutant of the muscle isozyme, which is insensitive to calcium ions while retaining all other kinetic properties resembling the liver isozyme, has been prepared and crystallized. Here, two crystal structures of this mutant enzyme in complex with AMP with and without fructose 6-phosphate (the product of the catalytic reaction) are presented. The AMP binding pattern of the muscle isozyme is quite similar to that of the liver isozyme and the T conformations of the two isozymes are nearly the same.

1. Introduction

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and orthophosphate (Benkovic & deMaine, 1982). The enzyme has been isolated, purified and characterized from numerous prokaryotes and eukaryotes (Tejwani, 1983). All known vertebrate FBPases are obligate homotetramers.

There are two genes for FBPase in mammals, coding for the liver and the muscle enzymes. The former is expressed in almost all mammalian tissues but predominantly in the liver and kidneys, while the latter is found only in muscle (Tillmann & Eschrich, 1998). The liver FBPase has been recognized as the regulatory enzyme of gluconeogenesis, but the physiological role of the muscle isozyme has remained a mystery for several decades. The lack of glucose-6-phosphatase activity in muscle excludes gluconeogenesis; nevertheless, glycogen synthesis is still possible (Ryan & Radziuk, 1995; Dzugaj, 2006). It has been postulated that muscle FBPase may participate in glycogen synthesis from carbohydrate precursors such as lactate. To distinguish it from gluconeogenesis, this pathway has been termed glyconeogenesis.

The basic kinetic properties of the muscle and liver FBPase isozymes are virtually identical. Both require divalent cations such as Mg^{2+}, Mn^{2+} or Zn^{2+} for activity and both are activated by K^+ and inhibited allosterically by AMP and competitively by fructose 2,6-bisphosphate (Van Schaftingen & Hers, 1981; Pilkis *et al.*, 1981). The main difference between the isozymes concerns their sensitivity towards AMP: the muscle enzyme is 35–100 times more sensitive than the liver isozyme. In addition, the muscle isozyme, unlike the liver isoform, is highly sensitive to inhibition by calcium ions (Gizak *et al.*, 2004). Site-directed mutagenesis experiments have shown that Glu69 is essential for the sensitivity of muscle FBPase towards calcium ions (Zarzycki *et al.*, 2007).

A concerted model of allosteric inhibition of liver FBPase by AMP has been postulated assuming two conformations of the enzyme: R (relaxed), which is active, and T (tense), which is inactive or only partially active (Tejwani, 1983). The allosteric properties of liver FBPase have been studied and the effects of pH, temperature and denaturing agents on the $I_{0.5}$ have been determined (Dzugaj *et al.*, 1976). Employing limited proteolysis, it has been shown that subtilisin cleaves the peptide bond between amino-acid residues 60 and 61 and that the N-terminal fragment of the enzyme remains associated with the remaining part of the protein. This limited proteolysis resulted in a lower sensitivity of the FBPase towards AMP (Dzugaj *et al.*, 1976). Consequently, it has been concluded that preservation of the intact structure is necessary to maintain the high affinity of FBPase towards AMP. To elucidate the structural details of these observations, crystallographic studies have been undertaken to map the localization and binding mode of the AMP molecule.

The first crystallographic study of FBPase was reported for the mammalian liver isozyme isolated from porcine kidney (Ke *et al.*, 1990; Zhang *et al.*, 1993). This structure has been extensively analyzed and the mechanisms of catalysis and allosteric inhibition by AMP have been elucidated. Each subunit (338 residues, ∼37 kDa) of the tetrameric enzyme binds one substrate and one AMP molecule at two distant sites located in clearly discernible domains (Ke *et al.*, 1991; Villeret *et al.*, 1995; Choe *et al.*, 2000). Binding of the substrate is accomplished with the participation of a loop comprised of residues 52–72, termed the 'dynamic loop'. This loop can assume three states, engaged, disordered and disengaged, the first two of which are attributed to the active enzyme. The mammalian liver FBPase consists of subunits labelled C1, C2, C3 and C4 and assembled as the 'upper' dimer (C1 and C2) and the 'lower' dimer (C3 and C4). During the R-to-T transition, several structural changes occur, such as a rearrangement of the AMP-binding domain and its 1.9° rotation towards the substrate-binding domain, coupled with conformational changes at the C1–C2 and C3–C4 interfaces, which lead to a 17–19° relative rotation of the dimers. The crystallographic studies also revealed the amino-acid residues involved in AMP binding (Ke *et al.*, 1990). It turned out that not only the AMP-binding residues but also residues from the 52–72 loop and from the C1–C2, C1–C3 and C1–C4 interfaces participate in the R-to-T transition (Choe *et al.*, 1998).

Although some preliminary crystallographic studies of the muscle isozyme have been reported (Zhu *et al.*, 2001), no details concerning the structure of the protein have been released. Therefore, we have undertaken a crystallographic analysis of muscle FBPase using a recombinant enzyme with a human muscle FBPase sequence containing the E69Q mutation.

The E69Q mutation abolishes the sensitivity of the muscle isozyme towards Ca^{2+} with concomitant preservation of all other kinetic properties, making it very similar to the liver isozyme. The only difference remains in the affinity towards AMP. In an effort to explain the origin of this dissimilarity, we have mapped the amino-acid residues involved in binding of the AMP inhibitor. Additionally, we have identified the amino-acid residues responsible for binding of the product of the catalytic reaction, fructose 6-phosphate (F6P), which in contrast to all other structures reported so far is found to be the α-anomer.

In this report, we present the first crystallographic study of muscle FBPase. Crystal structures of human muscle FBPase in the T conformation have been determined in complex with AMP (HMFBPI) as well as in complex with AMP and F6P (HMFBPIP). The product (F6P) and the AMP-binding sites are compared with the corresponding sites of human and porcine liver FBPases.

2. Methods

The mutagenesis and protein expression and purification experiments have been described in a previous report (Zarzycki *et al.*, 2007).

2.1. Protein crystallization

Before crystallization, the protein sample was diluted with Milli-Q water to a final buffer concentration of 20 m*M* and then concentrated to about 6 mg ml^{-1}. The crystallization experiments were carried out at 292 K in hanging drops using the vapour-diffusion method and the sparse-matrix screens Crystal Screen and Crystal Screen 2 from Hampton Research. 4 µl drops were made using a 1:1 volume ratio of the protein and precipitant solutions. The best crystals (0.25 × 0.12 × 0.10 mm) grew within five months over a reservoir consisting of 1.6 *M* ammonium sulfate, 0.1 *M* MES pH 6.5 and 10% dioxane.

2.2. Crystal soaking, data collection and processing

Two soaking experiments were performed using single crystals grown as above. In each soaking experiment the mother liquor was supplemented with 5 m*M* fructose 6-phosphate (F6P) or 5 m*M* adenosine monophosphate (AMP) with soaking times of 2.5 or 5 h, respectively. For data collection, the crystals were cryoprotected for a few seconds in the mother liquor supplemented with 25%(*v*/*v*) glycerol and, where appropriate, the above ligands and then flash-vitrified at 100 K in a cold nitrogen-gas stream.

Table 1
Data-collection and refinement statistics.

Treatment of crystals	No soaking (HMFBPI)	F6P soak (HMFBPIP)
Data collection		
Space group	*C*222	
Unit-cell parameters (Å)	a = 218.11, b = 234.26, c = 71.94	a = 218.14, b = 234.54, c = 71.75
Temperature (K)	100	
Radiation source	BESSY BL14.1	
Wavelength (Å)	0.91814	
Resolution (Å)	50.00–1.93 (2.01–1.93)	50.00–1.97 (2.04–1.97)
No. of observations	577747	514077
Unique reflections	128249	119283
$R_{\rm merge}$	0.059 (0.364)	0.070 (0.376)
$\langle I/\sigma(I)\rangle$	22.0 (2.1)	18.0 (2.1)
Completeness (%)	92.7 (57.2)	91.5 (48.8)
Multiplicity	4.5 (2.6)	4.3 (2.2)
Refinement		
Resolution (Å)	50.00–1.93 (2.03–1.93)	50.00–1.97 (2.08–1.97)
Reflections (work/test)	126899/1289	117923/1334
$R_{\rm work}/R_{\rm free}$	0.168/0.199	0.167/0.197
No. of atoms		
Protein	9909	9851
F6P		64
AMP	92	92
Water	650	702
$\langle B\rangle$ (Å^2)		
Protein	14.9	16.3
F6P		40.4
AMP	35.0	38.6
Water	20.1	21.0
R.m.s. deviations from ideal		
Bond lengths (Å)	0.0197	0.0197
Bond angles (°)	1.70	1.74
Ramachandran statistics (%)		
Most favoured	91.4	91.9
Additionally allowed	8.6	8.1
PDB code	3ifa	3ifc

X-ray diffraction data were collected from three crystals: (i) not soaked (1.93 Å), (ii) soaked with F6P (1.97 Å) and (iii) soaked with AMP (2.05 Å) using synchrotron radiation on beamline BL14.1 of the BESSY synchrotron, Berlin, Germany, and a Rayonics MX-225 3 × 3 mosaic CCD detector. The diffraction data were processed and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). The data-collection statistics are presented in Table 1.

2.3. Structure determination and refinement

The structure of the crystal that had not been soaked with any ligand was solved using the molecular-replacement program *MOLREP* (Vagin & Teplyakov, 2010) with the coordinates of one subunit of porcine liver FBPase (PDB code 1fj6; Nelson *et al.*, 2000) as the search model. The solution clearly showed four protein molecules in the asymmetric unit, combined into two pairs belonging to two different biologically relevant tetramers with crystallographic twofold symmetry. After several rounds of manual rebuilding in *Coot* (Emsley *et al.*, 2010), which included converting the model to the correct amino-acid sequence, interspersed with maximum-likelihood refinement in *REFMAC*5 (Murshudov *et al.*, 2011), the new model was transferred to the unit cells of the isomorphous crystals that had been subjected to ligand soaking. Difference electron-density maps for the F6P-soaked crystal clearly indicated the presence of this ligand in each subunit. At the same time, however, it turned out that each of the structures contained bound AMP, irrespective of the experimental approach regarding treatment with this ligand. It was therefore concluded that the presence of AMP was related to the purification procedure, in which a relatively high AMP concentration was used to release the protein from the chromatography column. It was further concluded that the association between the protein and the inhibitor must be very tight, as even thorough and prolonged dialysis runs did not wash the ligand out from the protein complex. Since all three structures contained bound AMP molecules, the final refinement of the FBPase–AMP complex (HMFBPI) was carried out only for the data set designated as 'not soaked' characterized by the highest resolution (1.93 Å). The other refined structure corresponds to the FBPase–AMP–F6P complex (HMFBPIP). The refinement, which included TLS parameterization as defined by the *TLSMD* server (Painter & Merritt, 2006), converged with R values of 0.168 and 0.167 ($R_{\rm free}$ values of 0.199 and 0.197) for the FBPase–AMP and FBPase–AMP–F6P complexes, respectively. The refinement statistics are presented in Table 1.

2.4. PDB accession codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 3ifa (FBPase–AMP complex; HMFBPI) and 3ifc (FBPase–AMP–F6P complex; HMFBPIP).

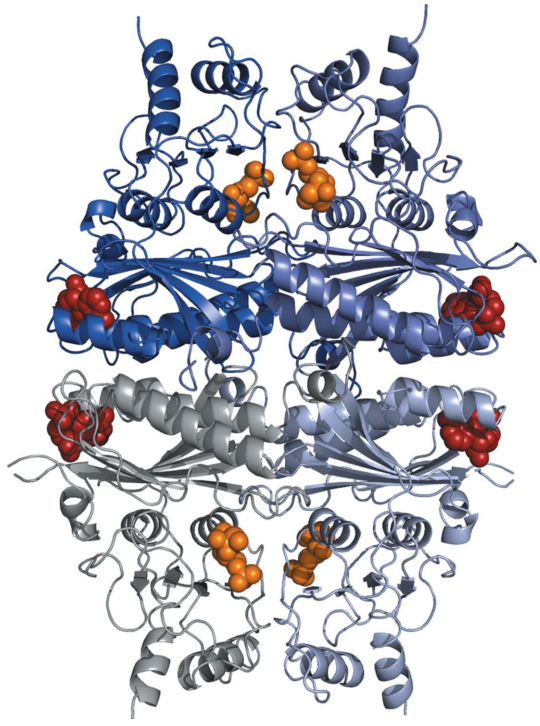


Figure 1
An overall view of a muscle FBPase (HMFBPIP structure) tetramer, represented as C1–C2–C3–C4 colour-coded subunits. The F6P (orange) and AMP (red) ligands are shown in van der Waals representation.

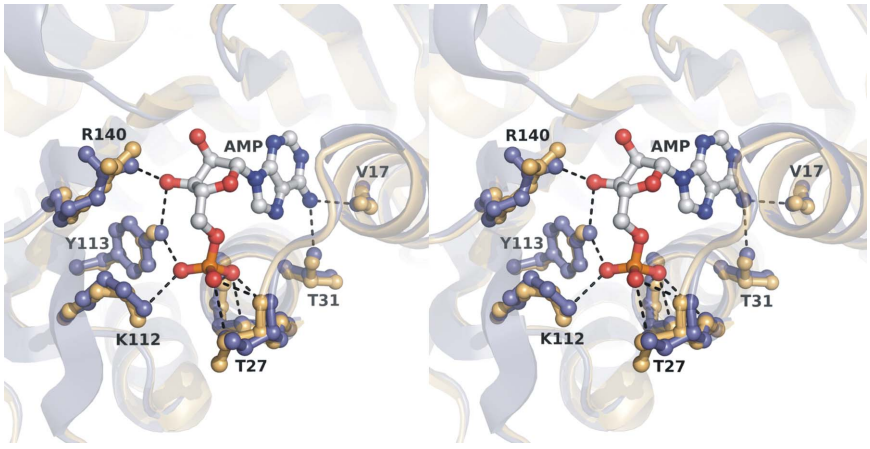


Figure 2
AMP-binding site of human muscle FBPase (HMFBPI, cyan) aligned with human liver FBPase (1fta, yellow) shown in ball-and-stick representation in stereoview. AMP is bound by residues Val17, Thr27, Thr31, Lys112, Tyr113 and Arg140 of subunit *A*. Hydrogen bonds are indicated for the muscle isozyme by broken lines.

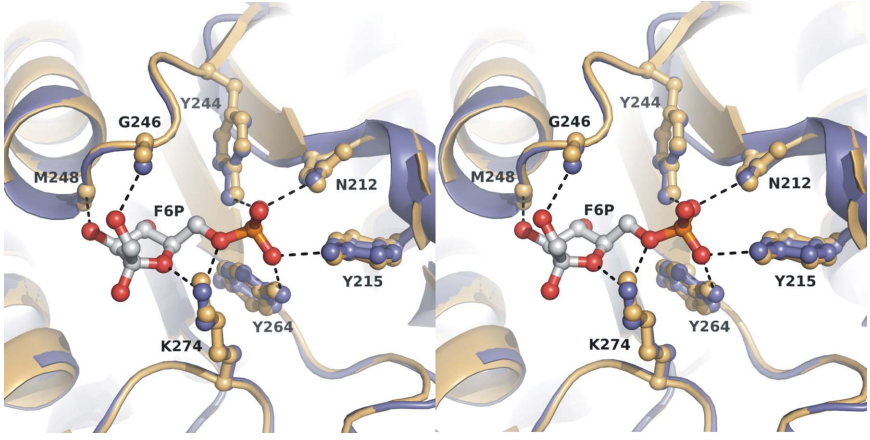


Figure 3
F6P-binding site of human muscle FBPase (HMFBPIP, cyan) aligned with porcine liver FBPase (1fbp, yellow; Ke *et al.*, 1990) shown in ball-and-stick representation in stereoview. F6P is bound by residues Asn212, Tyr215, Tyr244, Gly246, Met248, Tyr264 and Lys274 of subunit *A*. Hydrogen bonds are indicated for the muscle isozyme by broken lines.

3. Results

3.1. The crystal structure

The crystals of human muscle FBPase belonged to space group *C*222 and contained two independent dimers in the asymmetric unit situated at the crystallographic twofold axes along [010] (molecules *A*–*B*) and [100] (molecules *C*–*D*), which generate the complete C1–C2–C3–C4 [or, considering the crystallographic symmetry, *A*–*A*′–*B*–*B*′ (tetramer 1) and *C*–*C*′–*D*–*D*′ (tetramer 2)] biologically relevant tetramers (Fig. 1). The main-chain traces of the tetrameric models of the muscle isozyme generally follow the trace of the liver isozyme in the T conformation (PDB entries 1fta and 1eyj; Gidh-Jain *et al.*, 1994; Choe *et al.*, 2000). In these assemblies, the dynamic loop is in the disengaged conformation. Because of poor electron density, some amino-acid residues at the N-termini (residues 1–8) and in the dynamic loops (66–69) have not been modelled. Since tetramers 1 and 2 are highly similar, our further analysis concerning the structure of human muscle FBPase will refer to complex 2 unless stated otherwise.

3.2. The AMP-binding site

Each subunit of the FBPase binds one molecule of AMP at full occupancy. The nucleotide is mostly anchored at the phosphate group, which forms hydrogen bonds to the backbone N atoms of Thr27, Gly28, Glu29 and Leu30 and to the side chains of Thr27, Lys112 and Tyr113 (Fig. 2). In addition, the ribose ring is stabilized *via* hydrogen bonds between the O3′ hydroxyl group and the side chains of Tyr113 and Arg140. The exo-amino group of the adenine moiety interacts directly with the carbonyl O atom of Val17 and with the side chain of Thr31 and forms several other contacts mediated by water molecules. The residues involved in AMP binding in the present complex and in the complexes reported for human liver (1fta) and pig kidney (1eyj) FBPases are essentially the same (Fig. 2; Supplementary Table 1[1]).

3.3. The F6P-binding site

In the FBPase–AMP–F6P complex, each subunit of the muscle enzyme binds one molecule of fructose 6-phosphate at full occupancy. The ligand molecule is stabilized by a network of hydrogen bonds formed by the phosphate group with the side chains of Tyr215, Tyr244, Tyr264 and Asn212 and by the fructose hydroxyls O3′ and O6′ with the main-chain and side-chain N atoms of Met248 and Lys274, respectively (Fig. 3; Supplementary Table 2[1]). Depending on the protomer, none, one or two extra hydrogen bonds are created between O1′ and Gly246 and/or O5′ and Lys274. Additionally, each F6P molecule has a hydrogen-bond contact with the side chain of Arg243 from the second subunit within the upper and lower dimers.

In close proximity to the O1′ atom of each F6P molecule, a well ordered full-occupancy sulfate ion is present. It is stabilized in the active site by hydrogen bonds to the backbone N atom of Gly122 and the hydroxyl group of the Ser124 side chain. The sulfate ion, which originates from the crystallization buffer, is a chemical isostere of the phosphate group released from the fructose 1,6-bisphosphate substrate during the FBPase reaction (Fig. 4).

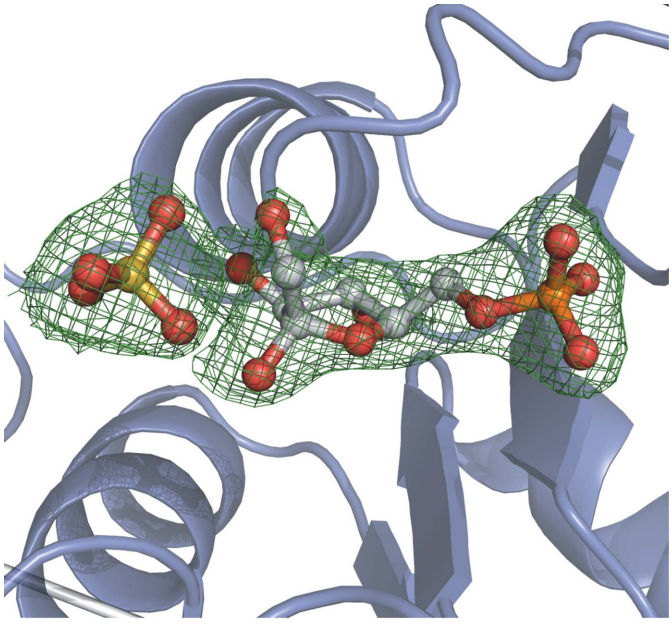
The substrate specificity of FBPase is a rather controversial issue. In solution, fructose 1,6-bisphosphate exists in an

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: DW5003). Services for accessing this material are described at the back of the journal.

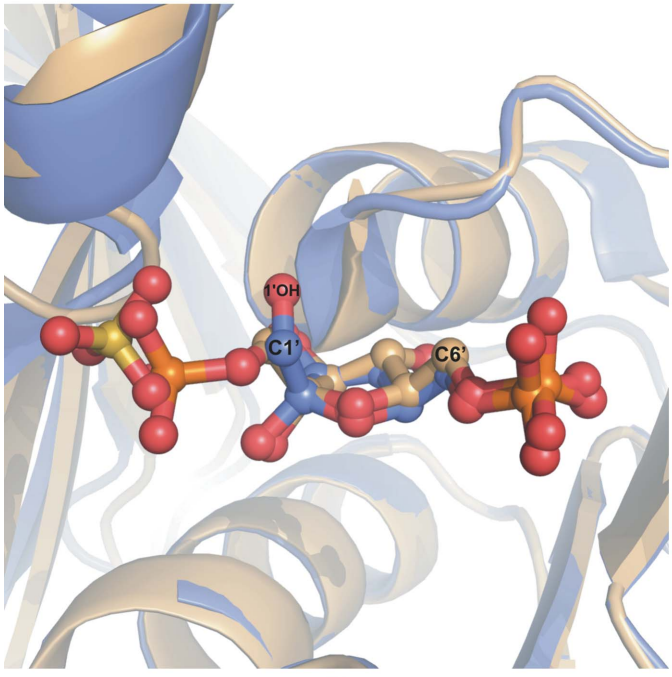
equilibrium between α-anomer (15%), β-anomer (81%), acyclic keto (2%) and *gem*-diol (1.3%) forms (Frey *et al.*, 1977). Based on equilibrium binding studies, it was claimed that 2,5-anhydro-D-mannitol 1,6-bisphosphate, an analogue of β-D-fructose 1,6-bisphosphate, is bound by the enzyme ten times more tightly than 2,5-anhydro-D-glucitol 1,6-bisphosphate, an analogue of α-D-fructose 1,6-bisphosphate (Marcus, 1976). As a conclusion, the β-anomer of fructose 1,6-bisphosphate has been interpreted as the real substrate of the enzyme. In contrast, employing rapid quench experiments, Benkovic & deMaine (1982) demonstrated the enzyme's stereospecificity for the α-anomer. They postulated that the β-anomer is utilized only after mutarotation to the α-form. In addition, Lipscomb and coworkers also postulated that the α-anomer of fructose 1,6-bisphosphate is indeed the substrate of FBPase (Villeret *et al.*, 1995). The electron density in the active site of the present complex of muscle FBPase is of superb quality and leaves no doubt that the F6P molecule is the α-anomer (Fig. 4*a*).

3.4. The dynamic loop

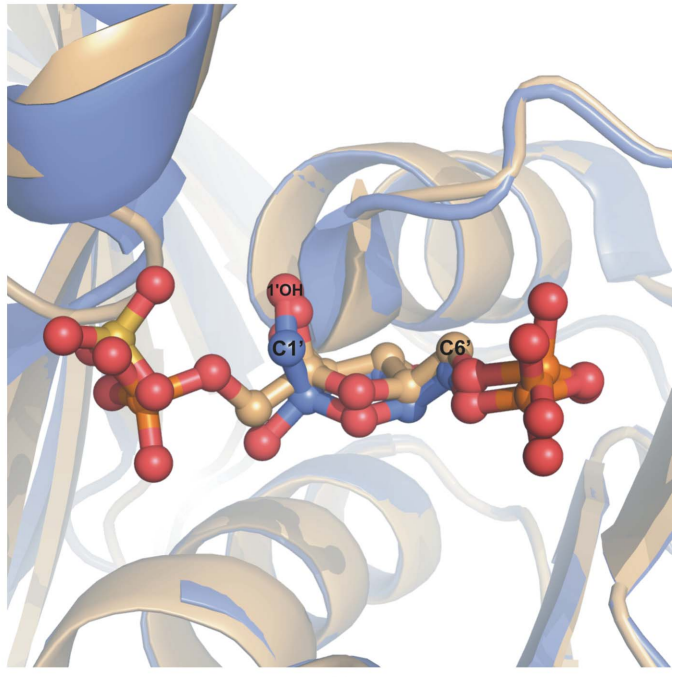
The dynamic 52–72 loop can assume three different states: engaged or disordered in the active tetramer or disengaged in the inactive form (Choe *et al.*, 1998). The dynamic loop in the present structure is in the 'disengaged' position, which is in agreement with previous reports showing a correlation of the loop conformation with the presence or absence of AMP and the T or R conformation of the tetramer. Not all of the amino-acid residues (including the E69Q mutation) are visible in the loop. Nevertheless, the disengaged conformation can be easily recognized (Fig. 5). We have previously shown (Zarzycki *et al.*, 2007) that the E69Q mutation has no effect on the binding of either AMP or the substrate. Therefore, this single-amino-acid substitution can be assumed to have negligible effect on the overall structure of the enzyme.



(*a*)



(*b*)



(*c*)

Figure 4
The product-binding site of human muscle FBPase. (*a*) shows the $F_o - F_c$ OMIT map (2.5σ contour) of the F6P ligand and the sulfate ion in the active site of muscle FBPase (HMFBPIP structure). (*b*) and (*c*) show the alignment of the substrate-binding regions of muscle (HMFBPIP, cyan) and liver (PDB entry 1fbh, yellow) FBPases. The F6P/sulfate ion (this work) is located in the vicinity of the 6′OH group of F6P and isosterically mimics the 1′-orthophosphate group of fructose 1,6-bisphosphate [shown in orange in (*b*) for the α-anomer and in (*c*) for the β-anomer].

4. Discussion

We have shown that the binding mode of AMP by muscle FBPase closely resembles the situation known from the liver FBPase–AMP complexes. The calculated C^{α} r.m.s. deviation (Gille & Frömmel, 2001; Zhang & Skolnick, 2005) between human (1fta) and porcine (1eyj) liver FBPases is 0.58 Å, compared with the value of 0.17 Å calculated for a superposition of the present complexes HMFBPIP and HMFBPI. The r.m.s.d. for the liver and muscle (this work) FBPase–AMP complexes is 0.73 Å and is slightly higher (0.96 Å) when the present FBPase–AMP–F6P complex is used. These increased values can be explained by the differences in the sequences of the liver and muscle isozymes.

In skeletal muscle, the inhibition of FBPase by AMP is of importance in the regulation of carbohydrate metabolism. An active FBPase in the presence of phosphofructokinase would result in a futile cycle. Under physiological conditions, the higher sensitivity of muscle FBPase towards AMP causes complete inhibition of the enzyme and protects the myocytes against unproductive waste of energy (Gizak *et al.*, 2008). The origin of the high sensitivity of muscle FBPase towards AMP has been investigated employing site-directed mutagenesis. Gizak *et al.* (2008) presented evidence that truncation starting from Pro5 at the N-terminus decreased the sensitivity of muscle FBPase towards AMP. Rakus *et al.* (2005) found that the three-point mutant K20E/T177M/Q179C was inhibited by AMP about 26 times more weakly than the wild-type muscle isozyme. They also constructed two chimeric human FBPases (L50M288 and M50L288; Rakus *et al.*, 2003) in which the N-terminal residues (1–50) were derived from the other isozyme. Ultimately, the authors concluded that there is a different method of AMP binding by the muscle isozyme in the R conformation.

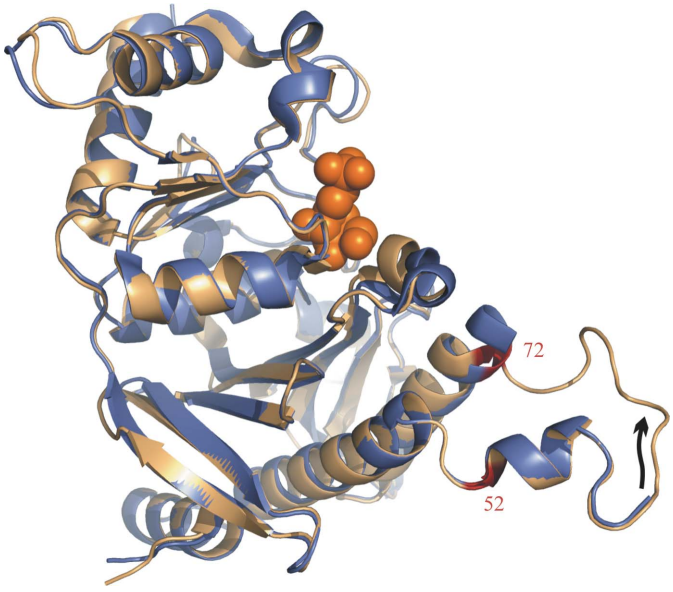


Figure 5
C^{α} superposition of muscle (HMFBPIP, cyan) and liver (1fbp, yellow) FBPases in the region of the dynamic loop and active site. F6P (orange) is shown in van der Waals representation. The amino-acid sequence of the loop is highly conserved. The gap in the HMFBPIP model is a consequence of poor electron density for residues 67–69. Despite this, the disengaged conformation of the loop can be recognized.

Considering the allosteric interaction of muscle FBPase with AMP, we took into account the concerted model of inhibition in which the two forms, R and T, are in equilibrium in the absence of any regulators. The inhibitor binds to the T form, shifting the equilibrium towards the T conformation. On the other hand, most reported studies have found that AMP binds to the R form of FBPase and that only this binding triggers the conformational changes leading to the T form. Therefore, the affinity for AMP gauged by the $I_{0.5}$ parameter reflects binding of the ligand by the enzyme in the R form. Since muscle FBPase is the more sensitive of the two isozymes to AMP inhibition, we may postulate that the R form of muscle FBPase differs significantly from the R form of the liver enzyme, enabling an R-to-T transition at a lower AMP concentration.

Additionally, we have shown that the reaction product fructose 6-phosphate is bound to the enzyme in its α configuration, despite the fact that the soaking solution contained an equilibrium mixture of the α- and β-anomers of D-fructofuranose 6-phosphate. Apparently, for some reason the muscle isozyme behaves differently to its liver analogue and appears to selectively prefer the opposite F6P anomer. Owing to the excellent electron-density maps at 1.9 Å resolution from the present structure determination, we could unambiguously demonstrate that the active site contains the α-anomer of F6P (Fig. 4).

This work was supported in part by grant No. NN301416333 from the Ministry of Science and Higher Education. Some of the calculations were carried out at the Poznan Metropolitan Supercomputing and Networking Center.

References

Benkovic, S. J. & deMaine, M. M. (1982). *Adv. Enzymol. Relat. Areas Mol. Biol.* **53**, 45–82.
Choe, J.-Y., Fromm, H. J. & Honzatko, R. B. (2000). *Biochemistry*, **39**, 8565–8574.
Choe, J.-Y., Poland, B. W., Fromm, H. J. & Honzatko, R. B. (1998). *Biochemistry*, **37**, 11441–11450.
Dzugaj, A. (2006). *Adv. Enzyme Regul.* **46**, 51–71.
Dzugaj, A., Chu, D. K., El-Dorry, H. A. & Horecker, B. L. (1976). *Biochem. Biophys. Res. Commun.* **70**, 638–646.
Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* D**66**, 486–501.
Frey, W. A., Fishbein, R., de Maine, M. M. & Benkovic, S. J. (1977). *Biochemistry*, **16**, 2479–2484.
Gidh-Jain, M., Zhang, Y., van Poelje, P. D., Liang, J.-Y., Huang, S., Kim, J., Elliott, J. T., Erion, M. D., Pilkis, S. J., el-Maghrabi, M. R. & Lipscomb, W. N. (1994). *J. Biol. Chem.* **269**, 27732–27738.
Gille, C. & Frömmel, C. (2001). *Bioinformatics*, **17**, 377–378.
Gizak, A., Maciaszczyk, E., Dzugaj, A., Eschrich, K. & Rakus, D. (2008). *Proteins*, **72**, 209–216.
Gizak, A., Majkowski, M., Dus, D. & Dzugaj, A. (2004). *FEBS Lett.* **576**, 445–448.
Ke, H. M., Liang, J. Y., Zhang, Y. P. & Lipscomb, W. N. (1991). *Biochemistry*, **30**, 4412–4420.
Ke, H. M., Zhang, Y. P. & Lipscomb, W. N. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 5243–5247.

Marcus, F. (1976). *FEBS Lett.* **70**, 159–162.
Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* D**67**, 355–367.
Nelson, S. W., Iancu, C. V., Choe, J.-Y., Honzatko, R. B. & Fromm, H. J. (2000). *Biochemistry*, **39**, 11100–11106.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Painter, J. & Merritt, E. A. (2006). *Acta Cryst.* D**62**, 439–450.
Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J. & Claus, T. (1981). *J. Biol. Chem.* **256**, 3619–3622.
Rakus, D., Maciaszczyk, E., Wawrzycka, D., Ułaszewski, S., Eschrich, K. & Dzugaj, A. (2005). *FEBS Lett.* **579**, 5577–5581.
Rakus, D., Tillmann, H., Wysocki, R., Ulaszewski, S., Eschrich, K. & Dzugaj, A. (2003). *Biol. Chem.* **384**, 51–58.
Ryan, C. & Radziuk, J. (1995). *Am. J. Physiol.* **269**, E538–E550.
Tejwani, G. A. (1983). *Adv. Enzymol. Relat. Areas Mol. Biol.* **54**, 121–194.
Tillmann, H. & Eschrich, K. (1998). *Gene*, **212**, 295–304.
Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* D**66**, 22–25.
Van Schaftingen, E. & Hers, H. G. (1981). *Proc. Natl Acad. Sci. USA*, **78**, 2861–2863.
Villeret, V., Huang, S., Fromm, H. J. & Lipscomb, W. N. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 8916–8920.
Zarzycki, M., Maciaszczyk, E. & Dzugaj, A. (2007). *FEBS Lett.* **581**, 1347–1350.
Zhang, Y., Liang, J. Y., Huang, S., Ke, H. & Lipscomb, W. N. (1993). *Biochemistry*, **32**, 1844–1857.
Zhang, Y. & Skolnick, J. (2005). *Nucleic Acids Res.* **33**, 2302–2309.
Zhu, D.-W., Xu, G.-J., Rehse, P., Shi, R., Zhao, F.-K. & Lin, S.-X. (2001). *Acta Cryst.* D**57**, 847–849.