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Structures of *S. pombe* phosphofructokinase in the F6P-bound and ATP-bound states

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Abstract

Phosphofructokinase (Pfk1; EC 2.7.1.11) is the third enzyme of the glycolytic pathway catalyzing the formation of fructose-1,6-bisphosphate from fructose-6-phosphate (F6P) and ATP.

Schizosaccharomyces pombe Pfk1 is a homo-octameric enzyme of 800 kDa molecular weight, distinct from its yeast counterparts which are mostly hetero-octameric enzymes composed of two different subunits. Having an “open” conformation and a tendency to aggregate into higher oligomeric structures, the *S. pombe* enzyme shows similarities to the mammalian muscle Pfk1. It has been proposed that due to the distinct N-terminal region of the *S. pombe* subunit, the oligomeric organization of subunits in this enzyme is different from other yeast phosphofructokinases.

Electron microscopy studies were carried out to reveal the quaternary structure of the homo-octameric Pfk1 from *S. pombe* in the F6P-bound and in the ATP-bound state. Random conical tilt data sets have been collected from deep stain preparations of the enzyme in both states. The 0° tilt images have been separated into different classes and a 3D reconstruction has been calculated for each class from the high tilt images. Our results confirm the presence of a variety of views of the particle, most of which can be interpreted as views of the molecule rotating around its long axis.

Despite the biochemical differences, the structure of phosphofructokinase from *S. pombe* in the presence of either F6P or ATP is similar to the hetero-octameric structure of phosphofructokinase from *Saccharomyces cerevisiae*. The molecule can be described as composed of two subdomains, connected by two well-defined densities. We have been able to establish a correlation between the kinetic behavior and the structural conformation of Pfk1.

Keywords

6-phosphofructokinase; *Schizosaccharomyces pombe*; glycolytic enzyme; fission yeast; random conical; 3D reconstruction; electron microscopy; simultaneous alignment; Radon transforms; CTF correction

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Introduction

Phosphofructokinase (Pfk1; EC 2.7.1.11) catalyzes the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate in the presence of ATP with the concomitant release of ADP. Catalyzing the first irreversible reaction specific for glycolysis, it logically plays a crucial role in the regulation of the entire pathway (Heinrich and Rapoport, 1974; Uyeda, et al., 1981; Hofmann and Kopperschläger, 1982; Kotlarz and Buc, 1982). Since Pfk1 was long accepted as the best representative for the Monod-Wyman-Changeux kinetic model for allosteric enzymes (Monod, et al., 1965), its kinetic properties have been studied in many organisms. In most species, Pfk1 shows a sigmoidal kinetic behavior for F6P with binding constants and effector regulation being species and tissue specific. The activity of prokaryotic Pfk1s is regulated by ADP in the role of activator and phosphoenol pyruvate as inhibitor. Eukaryotic Pfk1s have a more complex regulatory mechanism with more than 20 effectors (Sols, 1981). The substrate, ATP, which had not been shown to have an effect on the bacterial enzyme, has acquired, in higher organisms, an inhibitor role.

Bacterial Pfk1s are homo-tetramers composed of 35 kDa subunits. Eukaryotic enzymes have approximately double-sized subunits resulting from a gene duplication and fusion event (Poorman, et al., 1984; Heinisch, 1986). The genome of higher eukaryotes possesses three different Pfk1 isoforms of approximately 90 kDa each, which are present on the tetrameric enzyme at different stoichiometries in a tissue dependent fashion. The yeast kingdom shows greater complexity, and the Pfk1s exist in different oligomeric forms. Homo-oligomers are found in the non-fermentative red yeast *Rhodotorula glutinis* (tetrameric structure, (Schröter and Kopperschläger, 1996) and in the fission yeast *Schizosaccharomyces pombe* (octameric structure, (Reuter, et al., 2000). Other yeasts (*e.g. Saccharomyces cerevisiae* and *Kluyveromyces lactis*) form hetero-octameric Pfk1s composed of two different subunits (α - and β -subunits) of molecular weights about 100 kDa in a 1:1 stoichiometry (Kopperschläger, et al., 1977; Plietz, et al., 1978; Tijane, et al., 1979; Heinisch, et al., 1993; Bär, et al., 1997; Lorberg, et al., 1999; Reuter, et al., 2000).

Biochemical information is not sufficient to completely understand enzyme catalysis and regulation. Structural data available for the bacterial enzyme in different states has revealed the conformational changes undergone by the enzyme during catalysis (Evans, 1992). Additionally, in combination with kinetic data, it has shown that effectors act upon the enzyme by regulating its affinity for its substrates (Evans, 1992). A wealth of biochemical data exists for eukaryotic Pfk1s; however, structural information is only available for the baker's yeast (*S. cerevisiae*) in a reduced number of states. *S. cerevisiae* Pfk1 is an 835 kDa hetero-octamer, which shows cooperative binding for F6P and non-cooperative binding for ATP. The first indications of the subunit arrangement in the octameric structure were provided by small-angle X-ray scattering experiments (Plietz, et al., 1978) and by an electron microscopy interconversion study of 2D averages (Nissler, et al., 1985). Preliminary structures, showing the specific subunit organization and interactions, were calculated from random conical data sets from both negative stain- and cryo-preparations of the enzyme in the presence of F6P (Ruiz, et al., 2001). Later, the 3D structure of the F6P-bound state was obtained by cryo-electron microscopy to 10.8 Å resolution (Ruiz, et al., 2003). This electron microscopy structure, in combination with molecular replacement using the bacterial enzyme, provided the initial phases to solve the X-ray structure of the F6P-bound state of the 12S yeast truncated-tetramer (Mechin, 2002). The 3D reconstruction from frozen-hydrated preparations of the ATP-bound state of Pfk1 to 13 Å resolution shows that the structure in the presence of ATP is more swollen (Bárcena, et al., 2007). The calculated radius of gyration of 7.3 nm (7.0 nm for F6P) is in good agreement with SAXS data (Laurent, et al., 1984). Additionally, a substantial decrease in the rotational angle between

the top and bottom tetramers was observed, arising from a reorientation of the subunits in the dimers (Bárcena, et al., 2007).

S. pombe has a unique Pfk1 composed of eight identical subunits of 98 kDa molecular weight each (Reuter, et al., 2000). The amino acid sequence of the *S. pombe* Pfk1 subunit shows no specific similarity to the α - or β -subunit of other yeasts (49% and 47% identity to the α - and β -subunit of *S. cerevisiae*, respectively). In addition, the C-terminal domain of the molecule shows no strong similarity to the C-terminal domain of the α -subunit of other yeasts. The last 80 amino acids from the α -subunit of *S. cerevisiae* are implicitly involved in forming and maintaining the octameric structure (Edelmann, et al., 2002); the removal of these amino acids by proteolytic treatment results in the production of truncated tetramers (Kopperschläger, et al., 1993). *S. pombe* Pfk1 shows the highest sequence similarity, of all the yeast Pfk1s, to the human muscle enzyme (39% for the α - and β -subunits of *S. cerevisiae* and 44% for the *S. pombe* subunit). The muscle Pfk1 is an enzymatically active tetramer that can assemble into larger oligomers at high protein concentrations *in vitro*; a unique characteristic shared by the *S. pombe* Pfk1. In addition, the *S. pombe* Pfk1, like most purified yeast Pfk1s, is stable at low protein concentrations and starts dissociating at concentrations below 50 $\mu\text{g/ml}$ (Reuter, et al., 2000). Moreover, its kinetic behavior is different in its kingdom; it shows lower affinity and weaker cooperativity for F6P and lesser inhibition by ATP than *S. cerevisiae* Pfk1 (Reuter, et al., 2000). All these results suggested that the *S. pombe* enzyme was structurally different from other yeasts.

We have carried out electron microscopy studies of the Pfk1 from *S. pombe* in the presence of F6P and ATP to characterize the quaternary structure of the enzyme in the active-state (F6P-bound or R-state) and the inhibited-state (ATP-bound or T-state). Random conical tilt data sets were collected from deep stain preparations of the enzyme in each state. *S. pombe* Pfk1 in both the R- and the T-states can be described, similar to Pfk1 from *S. cerevisiae*, as a dimer of tetramers. In projection, it shows two distinct subdomains: a diamond shaped headpiece connected by two well-defined densities to a more rectangular shaped basepiece. Our results have proven the stability of the enzyme, and have shown the presence of a variety of views of the particle, which can be interpreted mostly as different views of the molecule rotating around its long axis and not as different conformations. However, we have observed that this enzyme preparation shows slightly higher structural variability than the *S. cerevisiae* Pfk1. The structural differences observed between the R- and T-states of the *S. pombe* and *S. cerevisiae* Pfk1s are correlated to their kinetic behavior. The results presented here are a further step to shed light into the structure/function relationship of Pfk1 of higher eukaryotes, particularly of the mammalian muscle enzyme.

Materials and methods

Enzyme purification

Phosphofructokinase from *Schizosaccharomyces pombe* was purified from the CBS-1057 strain, as described in Reuter *et al.* (2000) and stored in 30% glycerol at 4 °C until further use.

Electron microscopy

A small aliquot of the enzyme suspension was diluted to 5 mg/ml in 50 mM sodium phosphate buffer (pH 7.2), complemented with either 3 mM F6P or 1 mM ATP and 3 mM MgSO_4 , and run through a micro-Bio-Spin P30 column (BioRad). The enzyme was diluted to 20 $\mu\text{g/ml}$ in the presence of 25 $\mu\text{g/ml}$ of Tobacco Mosaic Virus (TMV), applied to 400 mesh copper grids coated with a thin film of carbon and deep stained with 1% uranyl acetate (Ruiz and Radermacher, 2006).

The grids were observed on a Tecnai T12 electron microscope (FEI, The Netherlands) equipped with a LaB6 cathode (Kimball, USA) and a 2048 × 2048 CCD camera (TVIPS, Germany). Images were recorded at an accelerating voltage of 100 kV, and 52,000X nominal magnification under low dose conditions on S0-163 Kodak film. The magnification was calibrated using TMV as a standard (53,500X). To acquire a random conical data set (Radermacher, et al., 1987; Radermacher, 1988), pairs of images from the same area were collected; the first at tilt angles ranging between 52°–60° and the second at 0°. The tilted images were collected with defoci ranging from 1.7 to 2.3 μm at the center of the negative, while the untilted images had defoci ranging from 1.1 to 1.5 μm . The electron dose was approximately 1000 e^-/nm^2 per image.

Image analysis

Image analysis was carried out using SPIDER (version 5.0, with modifications, (Frank, et al., 1996)), the WEB display program and XMIPP (Marabini, et al., 1996). Negatives were scanned with a 7 μm raster size on an Intergraph SCAI flatbed scanner (Z/I Imaging Corporation, Huntsville, AL). Micrographs were binned down by a factor of three, resulting in a calibrated pixel size of 0.39 nm on the sample scale. Particle coordinates were picked from tilt pairs using the command *Tilted Particles* in WEB. Selected particles were windowed with dimensions of 128×128 pixels and contrast normalized using the average density calculated within a ring surrounding the windowed particle. The data sets for Pfk1 in the F6P-bound state and in the ATP-bound state contained 5585 and 7039 particles, respectively.

The analysis of the random conical data sets essentially followed the procedure described in Ruiz *et al.* (2001), with the addition of correspondence analysis (van Heel and Frank, 1981; Frank and van Heel, 1982; Bretaudiere and Frank, 1986) and classification with moving centers (Diday, 1971) combined with hierarchical ascendant classification (HAC) (Radermacher, et al., 2006).

The contrast transfer function (CTF) of the microscope was fitted to all micrographs to obtain values for defocus and astigmatism. Both the windowed 0° and the tilted images were corrected using a smooth function to flip the phases (Radermacher, et al., 2001). The corrected images were interpolated to a pixel size 0.36 nm to facilitate comparison with earlier data sets of Pfk1 from other species. All alignments of the 0° images were carried out using the simultaneous translational/rotational alignment techniques based on the cross-correlation of Radon transforms (Radermacher, 1994; Radermacher, 1997). A first reference image was created by reference-free alignment (Marco, et al., 1996) and the complete image series was aligned to this reference. The aligned images were separated into groups of similar particles by an artificial neural network using XMIPP (Marabini, et al., 1996). The average images of each group were used as references in a multireference alignment procedure. Data sets underwent further processing by iterating correspondence analysis, followed by classification with Diday's method of moving centers and HAC (command *CLA* in SPIDER), and multireference alignment.

After the final alignment of the 0°-images, all the angles of the tilt-images are known. These include: the azimuthal angle from the alignment of the 0° images and the tilt angle defined by the data collection geometry, and accurately calibrated during the particle picking step. The header of the tilted images was updated with these angular parameters so that they could be used for calculating a 3D reconstruction per class. Two alternative techniques were used for centering the tilted images. Images were centered by cross-correlation with, either their matching 0° images that were foreshortened perpendicular to the tilt axis (F6P-bound state) or the corresponding 0° class averages foreshortened and oriented appropriately (ATP-bound state).

3D reconstructions were calculated using fast Radon inversion algorithms combined with projection onto convex sets (POCS) filters (Radermacher, 1997; Lanzavecchia, et al., 1999). This POCS algorithm imposes consistency on the noisy data and fills in data into the missing cones. The POCS algorithm was first applied to z slices (p, ϕ) and then to the y slices (p, θ). Filtering cycles were carried out until no further changes were detected in the Radon transform. The orientations of the projections were refined within each class by cross correlating the two-dimensional Radon transforms of the projections with the 3D Radon transform of the previously obtained volume (Radermacher, 1994). This procedure was iterated until the resolution of the volume was stable and minimal changes in the translation and angular orientation of the projections were found. After each refinement iteration, the new reconstruction was low-pass filtered to the resolution measured by Fourier Shell Correlation (FSC, (Saxton and Baumeister, 1982) with $5\times$ noise correlation criterion, and used as a new reference. To determine which classes could be merged, the volume calculated from the largest class was used as a reference to which all other volumes were compared, visually and numerically (Ruiz, et al., 2001; Bárcena, et al., 2007). For orientation alignment of similar volumes, the 0° projections of each of the volumes were Radon transformed and aligned to the 3D Radon transform of the reference. The volumes that had the smaller angular differences ($\sim 5^\circ$) were merged first; classes that showed larger angular differences ($\sim 20^\circ$) were merged later. Each merging step was followed by angular refinement of the merged projection set. The final merged volumes were refined until the resolution was stable. Volumes were visualized using Chimera (Pettersen, et al., 2004).

Results

S. pombe phosphofructokinase was originally preserved in ammonium sulfate at 80% saturation, which had given good results for the preservation of the *S. cerevisiae* enzyme. However, under these conditions, *S. pombe* Pfk1 showed a large number of higher molecular weight oligomers, both long strings of Pfk1 molecules and less ordered aggregates. We carried out combinations of biochemical and microscopy studies to find conditions to prevent or reduce the formation of larger aggregates. Our findings indicate that by replacing the ammonium sulfate at 80% saturation by 30% glycerol, in the last step of purification, preparations can be obtained where the enzyme is present mostly in the octameric form. Moreover, if stored under these conditions for a few months, the enzyme does not show any changes in its catalytic activity or in its aggregation behavior.

We have collected random conical data sets of *S. pombe* Pfk1 from deep stained preparations of the enzyme in the F6P-bound state (Fig. 1, left) and in the ATP-bound state (Fig. 1, right). At first glance, the enzyme looks similar to its *S. cerevisiae* counterpart (Ruiz, et al., 2001; Ruiz, et al., 2003; Bárcena, et al., 2007). However, in these preparations we have observed a few higher molecular weight oligomers (long strings of Pfk1 molecules, Fig. 1) that were never visualized in *S. cerevisiae* Pfk1 preparations. In addition, similar to the *S. cerevisiae* Pfk1 preparations, a small number of tetramers were found in these preparations. Pfk1 tetramers can be easily recognized by their distinct shape, two small squares located side by side, each square with a heavy stained center.

Self-organizing maps (Marabini, et al., 1996) were created from the 0° images of Pfk1 in both states, after one round of rotational/translational alignment to a reference calculated by reference-free alignment (data not shown). These maps allowed us to get an impression of the variability of views in the sample, and to select nodes to serve as references for the first round of multireference alignment. The final round of multireference alignment, correspondence analysis and classification of the 0° images produced 6 classes for the F6P-bound state (Fig. 2, bottom) and 12 classes for the ATP-bound state (Fig. 3, bottom). Figures 2 and 3 show examples of factor maps from the last correspondence analysis. Factor maps 1

vs 2 are depicted in both figures with factor 1 in the horizontal direction and factor 2 in the vertical direction (Figs. 2a and 3a). These are the most significant factors and represent the largest differences in the 0° images.

An analysis of the right-half of the F6P-bound state map (Fig. 2a) reveals similar projections as those found for the *S. cerevisiae* Pfk1 in the same state (Ruiz, et al., 2001). The molecule shows two distinct domains connected by two thin densities (central connectors) in a triangular conformation. The images on the bottom rows have very distinct squares in the bottom-half of the molecule, while the top-half shows weaker densities. These averages could be interpreted as a mixture of tetramers and not well-preserved molecules. The images in the top-left-quadrant are interesting because similar projections have been visualized in the ATP-bound state of *S. cerevisiae* Pfk1 where the central connectors have a rectangular conformation (Bárcena, et al., 2007). This suggested that *S. pombe* Pfk1 has a different angular rotation between the top and bottom tetramers. Similar views are observed in the ATP-bound state (Fig. 3). In this case, the putative tetrameric molecules are located at the left-half of the map (Fig. 3a), while the characteristic views for the *S. cerevisiae* ATP-bound state (central connectors in a rectangular conformation) are localized in the bottom-left-quadrant. Thus, for the F6P-bound state, the largest differences are between the rectangular and triangular conformations of the central connectors (factor 1) and the putative tetramers (factor 2). Factor 3 (Fig. 2b, vertical) can be interpreted as views of the molecule rotating along its long axis. Similar differences are represented in figure 3 for the ATP-bound state.

3D reconstructions were calculated from the tilted image-pairs and the position of the projections was refined until the rotational and translational parameters and the resolution were stable. Class 2 (Fig. 2), containing ~10% of the data set, gave rise to a structure with low resolution where the top and bottom regions of the molecule were not connected. The 3D structures from classes 1, 4–6 of the F6P-bound state were all very similar and represented views of the molecule in different orientations. These volumes were merged and the rotational/translational position of their projections was further refined (Fig. 4). Class 3, containing 7% of the data, showed a well-connected molecule, however, it was not merged with the rest of the classes because the top and bottom tetramers showed a slightly different orientation. For the ATP-bound state, the tetramers and not-so-well preserved molecules were sorted into class 12 (Fig. 3), which contained 912 images ~13% of the data set. Merging of classes 8–10 showed a well-preserved molecule, however at an orientation very different from the volumes of the other classes. Thus, they were not merged with the rest of the classes. Classes 1–7 and 11 were all merged and refined, since, as in the case of the F6P-state, the volumes showed different views of the molecule that differed only by a small angle (Fig. 5).

The 3D reconstructions shown in figures 4 and 5 have been calculated from 4100 and 4700 images of the enzyme in the F6P-bound and ATP-bound state, respectively. The final resolutions for the merged volumes are 2.5 nm for the F6P-bound state and 2.6 nm for the ATP-bound state using the 0.3 FSC criterion (Fig. 6, (Rosenthal and Henderson, 2003)). *S. pombe* Pfk1 in the F6P-bound state is 19 nm long and 11 nm wide (Fig. 4), while in the ATP-state it is 21 nm long and 14 nm wide (Fig. 5). In both, the F6P-bound (Fig. 4) and the ATP-bound (Fig. 5) states, the enzyme can be described, similar to Pfk1 from *S. cerevisiae*, as a dimer of tetramers (Figs. 4 and 5, top-tetramer in yellow and bottom-tetramer in blue). Both, in projection and in the central section of the volume, the molecule shows a diamond-shaped headpiece connected by two central connectors to a more rectangular shaped basepiece. Within the headpiece, there are two high density regions that extend outwards as lower densities. As it was the case for *S. cerevisiae*, only the basepiece is subdivided into two domains that can be interpreted as dimers of subunits, while no subdivisions are observed in the headpiece. Although octameric Pfk1s are open or loose structures and

always show a certain degree of flattening in negatively stained preparations, the *S. pombe* molecule in both states looks more flattened than its *S. cerevisiae* counterpart (Ruiz, et al., 2001). This effect may be due to weaker interactions between the top and the bottom tetramers. We measured the rotation angle between the top and the bottom tetramers and found a 65° rotation angle for the F6P-bound state and 50° for the ATP-bound state.

Discussion

We have carried out electron microscopy studies of Pfk1 from *S. pombe* to characterize the quaternary structure of the enzyme in the active and inhibited states. The working hypothesis was that the quaternary structure of *S. pombe* Pfk1 differs from the one of *S. cerevisiae*, which can be described as a dimer of tetramers. Several pieces of genetic and biochemical evidence supported this claim: a) *S. pombe* has a single Pfk1 subunit, b) the amino acid sequence shows no strong similarity to the α - or β -subunit of other yeasts, c) *S. pombe* Pfk1 shows a different kinetic behavior than other yeasts Pfk1's (Reuter, et al., 2000). In summary, all these data seemed to indicate that the oligomeric organization of the subunits was different from their yeast counterparts.

Our results show that the quaternary structure of *S. pombe* Pfk1 is octameric in both the F6P-bound and the ATP-bound states. At the low concentrations used for our experiments (~20 $\mu\text{g/ml}$), most of the enzyme is in the octameric form; only ~10% of the molecules had disassembled into tetramers. At low resolution, both the *S. cerevisiae* and the *S. pombe* enzymes are alike, and consist of a dimer of tetramers. In addition, the shape of the subunits (Figs. 4 and 5) and the subunit arrangement are similar. It is easy to distinguish, in *S. pombe* Pfk1, the L-shaped subunits that had originally been described for *S. cerevisiae* from an electron microscopy interconversion study of 2D averages of negatively stained preparations (Nissler, et al., 1985) and confirmed in a 3D study from frozen-hydrated preparations (Ruiz, et al., 2001). Each arm of the L-shaped subunits shows two subdomains like the *E. coli* subunit (Figs. 4 and 5, small subdomains labeled with # and large subdomains with *). The relative orientation of the L-shaped subunits seems to be identical to the one for *S. cerevisiae*. However, it is difficult to discern with our present data if the interactions between the subunits of a dimer forming the catalytic surface are also similar. In the F6P state of *S. cerevisiae* Pfk1, the small subdomain of the N-terminal domain of the α -subunit interacts with the large subdomain of the N-terminal region of the β -subunit; while in the ATP-bound state, Pfk1 changes its conformation and the subunits make stronger interactions through the small subdomains (Ruiz, et al., 2001; Ruiz, et al., 2003; Bárcena, et al., 2007). For *S. pombe* Pfk1, in both the F6P-bound and the ATP-bound states the interactions seem to be closer to those of *S. cerevisiae* in the ATP-bound state, or slightly stronger between the small (Figs. 4 and 5, #), and the large subdomains (Figs. 4 and 5, *) in the F6P-bound state. Even though we cannot determine the specific nature of the interaction and its strength, a strong indication that the R- and T-states of *S. pombe* are structurally in between the R- and T-states of *S. cerevisiae* comes from the observed rotation angle between the two tetramers. In *S. pombe* the angles are 65° for the F6P-bound state and 50° for the ATP-bound state, compared to 75° and 46°, respectively, for the F6P-bound and ATP-bound states of *S. cerevisiae* Pfk1. We have shown that the angular orientation between the two tetramers of *S. cerevisiae* Pfk1 is intimately correlated with the position of the subunits in the dimer forming the catalytic surface (Bárcena, et al., 2007). At smaller angles, the subunits show stronger interactions between the small subdomains making catalysis unfavorable. Thus, we can infer from our data that in the R-state the catalytic surface of *S. pombe* Pfk1 shows less overlap between the large (Fig. 4, *) and the small (Fig. 4, #) subdomains than in *S. cerevisiae* Pfk1. Similarly, we can infer that in the T-state, the *S. pombe* small (Fig. 5, #) and large (Fig. 5, *) subdomains show stronger overlap than in *S. cerevisiae* Pfk1. In addition, the molecule is larger in the ATP-bound state than in the F6P-bound state as was its *S.*

cerevisiae counterpart. All these results are in good agreement with the kinetic data that shows lower affinity and weaker cooperativity for F6P and lesser inhibition by ATP than *S. cerevisiae* Pfk1 (Reuter, et al., 2000)

In summary, we have determined the quaternary structure of *S. pombe* phosphofructokinase, and have demonstrated that it is an octamer, which consists of a dimer of tetramers the same as the *S. cerevisiae* Pfk1. We have shown that the preparations are homogeneous to 90% in both states; and that most of the variations observed in the projections can be explained as different views of the molecules rotating along their long axis. This will allow us to carry out higher resolution studies using similar methods as those used for analyzing the structure of *S. cerevisiae* Pfk1. In addition, the molecular dimensions, and the angular rotation between the tetramers indicate a similar behavior of the *S. pombe* and *S. cerevisiae* enzymes in the R-state (F6P-bound) and T-state (ATP-bound). The differences in the relative rotation of the tetramers in the R- and T-states of the two species show good correlation with their kinetic behavior. Thus, showing a strong link between structural conformation and function for Pfk1, which can be exploited in future studies of the enzyme. These results bring us a step closer to achieve a better understanding of the structural/functional relationship of the eukaryotic phosphofructokinase family.

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References

- Bär J, Schellenberger W, Kopperschlager G. Purification and characterization of phosphofructokinase from the yeast *Kluyveromyces lactis*. *Yeast*. 1997; 13:1309–17. [PubMed: 9392075]
- Bárcena M, Radermacher M, Bär J, Kopperschlager G, Ruiz T. The structure of the ATP-bound state of *S. cerevisiae* phosphofructokinase determined by cryo-electron microscopy. *Journal of Structural Biology*. 2007; 159:135–143. [PubMed: 17493831]
- Bretaudiere JP, Frank J. Reconstitution of molecule images analysed by correspondence analysis: a tool for structural interpretation. *J Microsc*. 1986; 144:1–14. [PubMed: 3632765]
- Diday E. La methode des nuees dynamiques. *Rev Stat Appl*. 1971; 19:19–34.
- Edelmann A, Kirchberger J, Heinisch JJ, Kopperschlager G. C-terminal modification of 6-phosphofructo-1-kinase from *Saccharomyces cerevisiae* and its influence on enzyme structure and activity. *Biochemical & Biophysical Research Communications*. 2002; 295:992–9. [PubMed: 12127994]
- Evans, PR. Activity and allosteric regulation in bacterial phosphofructokinase. *Proceedings of the Robert A. Welch Foundation Conference on Chemical Research XXXVI. Regulation of Proteins by Ligands*; Houston, Texas. 1992. p. 139-154.
- Frank J, Radermacher M, Penczek P, Zhu J, Li Y, Ladjadj M, Leith A. SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *Journal of Structural Biology*. 1996; 116:190–9. [PubMed: 8742743]
- Frank J, van Heel M. Correspondence analysis of aligned images of biological particles. *J Mol Biol*. 1982; 161:134–7. [PubMed: 7154073]
- Heinisch J. Isolation and characterization of the two structural genes coding for phosphofructokinase in yeast. *Molecular & General Genetics*. 1986; 202:75–82. [PubMed: 3007939]
- Heinisch J, Kirchrath L, Liesen T, Vogelsang K, Hollenberg CP. Molecular genetics of phosphofructokinase in the yeast *Kluyveromyces lactis*. *Mol Microbiol*. 1993; 8:559–70. [PubMed: 8326866]

- Heinrich R, Rapoport TA. A linear steady-state treatment of enzymatic chains. General properties, control and effector strength. *European Journal of Biochemistry*. 1974; 42:89–95. [PubMed: 4830198]
- Hofmann E, Kopperschlager G. Phosphofructokinase from yeast. *Methods in Enzymology*. 1982; 90:49–60. [PubMed: 6218374]
- Kopperschlager G, Bär J, Nissler K, Hofmann E. Physicochemical parameters and subunit composition of yeast phosphofructokinase. *European Journal of Biochemistry*. 1977; 81:317–25. [PubMed: 145942]
- Kopperschlager G, Bär J, Stellwagen E. Limited proteolysis of yeast phosphofructokinase. Sequence locations of cleavage sites created by the actions of different proteinases. *European Journal of Biochemistry*. 1993; 217:527–33. [PubMed: 8223596]
- Kotlarz D, Buc H. Phosphofructokinases from *Escherichia coli*. *Methods in Enzymology*. 1982; 90:60–70. [PubMed: 6218375]
- Lanzavecchia S, PLB, Radermacher M. Fast and Accurate Three-dimensional Reconstruction from Projections with Random Orientations via Radon transforms. *Journal of Structural Biology*. 1999; 128:152–164. [PubMed: 10600569]
- Laurent M, Tijane MN, Roucoux C, Seydoux FJ, Tardieu A. Solution X-ray scattering studies of the yeast phosphofructokinase allosteric transition. Characterization of an ATP-induced conformation distinct in quaternary structure from the R and T states of the enzyme. *Journal of Biological Chemistry*. 1984; 259:3124–6. [PubMed: 6230356]
- Lorberg A, Kirchrath L, Ernst JF, Heinisch JJ. Genetic and biochemical characterization of phosphofructokinase from the opportunistic pathogenic yeast *Candida albicans*. *European Journal of Biochemistry*. 1999; 260:217–26. [PubMed: 10091602]
- Marabini R, Masegosa IM, San Martin MC, Marco S, Fernandez JJ, de la Fraga LG, Vaquerizo C, Carazo JM. Xmipp: An Image Processing Package for Electron Microscopy. *J Struct Biol*. 1996; 116:237–40. [PubMed: 8812978]
- Marco S, Chagoyen M, de la Fraga LG, Carazo JM, Carrascosa JL. A Variant to the Random Approximation of the Reference-free Alignment Algorithm. *Ultramicroscopy*. 1996; 66:5–10.
- Mechin, I. PhD Thesis. Dept. Biochemistry, Leipzig University; 2002. Crystal Structure of Fructose-6-Phosphate Liganded R-State 6-Phosphofructo-1-Kinase from *Saccharomyces cerevisiae* at 2.9 Å Resolution.
- Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: a plausible model. *Journal of Molecular Biology*. 1965; 12:88–118. [PubMed: 14343300]
- Nissler K, Hofmann E, Stel'maschuk V, Orlova E, Kiselev N. An electron microscopy study of the quaternary structure of yeast phosphofructokinase. *Biomedica Biochimica Acta*. 1985; 44:251–9. [PubMed: 2408613]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera - A Visualization System for Exploratory Research and Analysis. *Journal of Computational Chemistry*. 2004; 25:1605–1612. [PubMed: 15264254]
- Plietz P, Damaschun G, Kopperschlager G, Müller JJ. Small-angle x-ray scattering studies on the quaternary structure of phosphofructokinase from baker's yeast. *FEBS Letters*. 1978; 91:230–2. [PubMed: 150337]
- Poorman RA, Randolph A, Kemp RG, Heinrikson RL. Evolution of phosphofructokinase--gene duplication and creation of new effector sites. *Nature*. 1984; 309:467–9. [PubMed: 6233492]
- Radermacher M. Three-dimensional reconstruction of single particles from random and nonrandom tilt series. *Journal of Electron Microscopy Technique*. 1988; 9:359–94. [PubMed: 3058896]
- Radermacher M. Three-dimensional reconstruction from random projections: orientational alignment via Radon transforms. *Ultramicroscopy*. 1994; 53:121–36. [PubMed: 8171751]
- Radermacher M. Radon transform techniques for alignment and 3D reconstruction from random projections. *Scanning Microscopy*. 1997; 11:171–177.
- Radermacher M, Ruiz T, Clason T, Benjamin S, Brandt U, Zickermann V. The three-dimensional structure of complex I from *Yarrowia lipolytica*: a highly dynamic enzyme. *J Struct Biol*. 2006; 154:269–79. [PubMed: 16621601]

- Radermacher M, Ruiz T, Wieczorek H, Grüber G. The structure of the V1-ATPase determined by Three-Dimensional Electron Microscopy of single particles. *Journal of structural biology*. 2001; 135:26–37. [PubMed: 11562163]
- Radermacher M, Wagenknecht T, Verschoor A, Frank J. Three-dimensional reconstruction from a single-exposure, random conical tilt series applied to the 50S ribosomal subunit of *Escherichia coli*. *Journal of Microscopy*. 1987; 146:113–36. [PubMed: 3302267]
- Reuter R, Naumann M, Bär J, Haferburg D, Kopperschlager G. Purification, molecular and kinetic characterization of phosphofructokinase-1 from the yeast *Schizosaccharomyces pombe*: evidence for an unusual subunit composition. *Yeast*. 2000; 16:1273–1285. [PubMed: 11015725]
- Rosenthal PB, Henderson R. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. *J Mol Biol*. 2003; 333:721–45. [PubMed: 14568533]
- Ruiz T, Kopperschlager G, Radermacher M. The first three-dimensional structure of phosphofructokinase from *Saccharomyces cerevisiae* determined by electron microscopy of single particles. *Journal of Structural Biology*. 2001; 136:167–80. [PubMed: 12051897]
- Ruiz T, Mechin I, Bär J, Rypniewski W, Kopperschlager G, Radermacher M. The 10.8 Å structure of *Saccharomyces cerevisiae* phosphofructokinase determined by cryoelectron microscopy: localization of the putative fructose 6-phosphate binding sites. *J Struct Biol*. 2003; 143:124–34. [PubMed: 12972349]
- Ruiz T, Radermacher M. Three-dimensional analysis of single particles by electron microscopy: sample preparation and data acquisition. *Methods Mol Biol*. 2006; 319:403–25. [PubMed: 16719366]
- Saxton WO, Baumeister W. The correlation averaging of a regularly arranged bacterial cell envelope protein. *Journal of Microscopy*. 1982; 127:127–138. [PubMed: 7120365]
- Schröter A, Kopperschlager G. 6-phosphofructo-1-kinase from the lipid accumulating, non-fermentative, red yeast *Rhodotorula glutinis*. *FEMS Microbiology Letters*. 1996; 142:247–52. [PubMed: 8810508]
- Sols A. Multimodulation of enzyme activity. *Current Topics in Cellular Regulation*. 1981; 19:77–101. [PubMed: 6460594]
- Tijane MN, Seydoux FJ, Hill M, Roucoux C, Laurent M. Octameric structure of yeast phosphofructokinase as determined by crosslinking with disuccinimidyl beta-hydromuconate. *FEBS Letters*. 1979; 105:249–53. [PubMed: 158542]
- Uyeda K, Furuya E, Sherry AD. The structure of “activation factor” for phosphofructokinase. *Journal of Biological Chemistry*. 1981; 256:8679–84. [PubMed: 6455427]
- van Heel M, Frank J. Use of multivariate statistics in analysing the images of biological macromolecules. *Ultramicroscopy*. 1981; 6:187–94. [PubMed: 7268930]

Figure 1. Random Conical Tilt Pairs

Left: Tilt pair for the F6P-bound state data set. Scale bar: 100 nm. with extracted 0° images displayed below. Scale bar: 10 nm. Right: Tilt pair for the ATP-bound state Scale bar: 100 nm, with extracted 0° images displayed below. Scale bar: 10 nm.

Figure 2. Factor Maps and Class Averages

F6P-bound state: a) maps of factors 1 and 2. The horizontal axis is factor 1 and the vertical axis factor 2. b) maps of factors 2 and 3. The horizontal axis is factor 2 and the vertical axis factor 3. Bottom row: Class averages from the last classification step. Indicated is the number of images included in the average. Scale bar: 10 nm.

Figure 3. Factor Maps and Class Averages

ATP-bound state: a) maps of factors 1 and 2. The horizontal axis is factor 1 and the vertical axis factor 2. b) maps of factors 3 and 4. The horizontal axis is factor 3 and the vertical axis factor 4. Bottom rows: Class averages from the last classification step. Indicated is the number of images included in the average. Scale bar: 10 nm.

Figure 4. Z-Slices and Surface Representation

Top panel: Z-slices through the merged reconstruction for F6P-bound state, spaced by 3.6 nm. Scale bar 10 nm. Bottom panel: Surface representations visualized in Chimera. The top-tetramer is shown in yellow and the bottom-tetramer in blue. The small subdomains of the subunits are labeled with # and the large subdomains with *. To the right is the same surface without labels. Bottom row: left) volume turned by 45°, right) volume turned by 135°. Scale bar: 10 nm.

Figure 5. Z-Slices and Surface Representation

Top panel: Z-slices through the merged reconstruction for ATP-bound state, spaced by 3.6 nm. Scale bar 10 nm. Bottom panel: Surface representations visualized in Chimera. The top-tetramer is shown in yellow and the bottom-tetramer in blue. The small subdomains of the subunits are labeled with # and the large subdomains with *. To the right is the same surface without labels. Bottom row: left) volume turned by 45°, right) volume turned by 135°. Scale bar 10; nm.

Figure 6. Resolution Measurements

Fourier shell correlation curves (F6P-bound state, bold line) and (ATP-bound state, dashed line), 5 times noise correlation curve (solid line).