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3D structure of phosphofructokinase from *Pichia pastoris*: localization of the novel γ -subunits

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Abstract

The largest and one of the most complex ATP-dependent allosteric phosphofructokinase (Pfk) has been found in the methylotrophic yeast, *Pichia pastoris*. The enzyme is a hetero-oligomer (~ 1 MDa) composed of three distinct subunits (α , β and γ) with molecular masses of 109, 104 and 41 kDa, respectively. While the α - and β -subunits show sequence similarities to other phosphofructokinase subunits, the γ -subunit does not show high homology to any known protein in the databases. We have determined the first quaternary structure of *P. pastoris* phosphofructokinase by 3D electron microscopy. Random conical techniques and tomography have been instrumental to ascertain the quality of the sample preparations for structural studies and to obtain a reliable 3D structure. The final reconstruction of *P. pastoris* Pfk resembles its yeast counterparts with four additional densities, assigned to four γ -subunits, bridging the N-terminal domains of the four pairs of α - and β -subunits. Our data has evidenced novel interactions between the γ - and the α -subunits comparable in intensity to the interactions, shown by cross-linking and limited proteolytic degradation experiments, between the γ - and β -subunits. The structural data provides clear insights into the allosteric fine-tuned regulation of the enzyme by ATP and AMP observed in this yeast species.

Keywords

Image processing; 6-phosphofructokinase; glycolysis; glycolytic enzyme; yeast; eukaryotic; random conical; tomography; 3D reconstruction; electron microscopy; simultaneous alignment; Radon transforms; CTF correction

Introduction

The conversion of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate with the concomitant hydrolysis of adenosine triphosphate (ATP) represents the first irreversible step specific for glycolysis. This reaction, catalyzed by phosphofructokinase (Pfk; 6-

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phosphofructokinase, EC 2.7.1.11), is subjected to tight control, thus rendering it a critical regulatory point of the glycolytic flux (Hofmann, 1976; Heinrich, et al., 1977; Sols, 1981). In prokaryotes, the activity of Pfk is mainly controlled by two effectors adenosine diphosphate and phosphoenolpyruvate. In contrast, more than 20 effectors (including ATP, adenosine monophosphate (AMP), fructose 2,6-bisphosphate (F26bP) and citrate) influence the activity of the eukaryotic counterparts (Sols, 1981). The increased degree of regulation in eukaryotes is due to duplication, fusion and mutation events of the ancestral bacterial gene, which have given rise to “double-size” eukaryotic genes containing additional regulatory sites (Poorman, et al., 1984; Heinisch, et al., 1989). The appearance of the second Pfk yeast gene resulted from a second duplication event, which occurred after the branching of *S. pombe* and *Y. lipolytica* in the phylogenetic tree. The variety of oligomerization states and kinetic properties of the active enzyme contribute an additional level of complexity to the enzyme. Eukaryotic Pfk can assemble into heterologous tetramers and larger homo- and hetero-oligomers, in contrast to the canonical homotetrameric form found in prokaryotes. Yeasts present the largest diversity; homotetramers in *Rhodotorula glutinis* (Schröter and Kopperschlager, 1996); homo-octamers in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* (Reuter, et al., 2000; Flores, et al., 2005); hetero-octamers in *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Candida albicans* (Kopperschlager, et al., 1977; Tijane, et al., 1979; Bär, et al., 1997; Lorberg, et al., 1999); and recently it was discovered that *Pichia pastoris* Pfk forms either heterododecamers or -tetradecamers (Tanneberger, et al., 2007).

P. pastoris Pfk has a sedimentation coefficient $s_{20,c} = 24$ S and a molecular mass of 975 kDa. It is the largest and one of the more complex representatives of the phosphofructokinase family (Kirchberger, et al., 2002). In addition, it possesses a unique structural organization. The enzyme is composed of three distinct subunits -- α , β and γ -- of 109 kDa, 104 kDa and 41 kDa, respectively, encoded by the *PFK1*, *PFK2* and *PFK3* genes (Edelmann and Bar, 2002; Tanneberger, et al., 2007). The α -subunit (encoded by *PFK1*) was originally identified due to its involvement in the initial steps of peroxisomal microautophagy (Yuan, et al., 1997). Remarkably, the ability of the α -subunit to modulate glucose-induced microautophagy is independent of its ability to metabolize sugars, since a catalytically inactive *PFK1* gene product can rescue the microautophagy phenotype (Yuan, et al., 1997). The α - and β -subunits share sequence similarities with the α - and β -subunits of other hetero-oligomeric yeasts. The γ -subunit has a distinct sequence and no protein homologue has been found in the accessible databases. However, biochemical studies confirm that it is an integral component of the *P. pastoris* Pfk oligomer. The kinetic behavior of *P. pastoris* Pfk is characteristic of a typical allosteric ATP-dependent phosphofructokinase, showing similar cooperativity to F6P as *S. cerevisiae* Pfk, but an increased sensitivity to ATP inhibition. In contrast to other members of the family, *P. pastoris* Pfk reacts more strongly to AMP activation than to activation by F26bP. Efforts to determine the specific role of the novel γ -subunit have concluded that this subunit is not required for the catalytic process (Tanneberger, et al., 2007). The γ -subunit is mainly involved in the allosteric fine tuning of the enzymatic activity, thus contributing to the adaptation of the organism to changes in the environmental conditions. The specific oligomeric organization has been inferred from results of genetic and biochemical experiments (Kirchberger, et al., 2002; Tanneberger, et al., 2007). These studies have led to a model, with a $4\alpha:4\beta:4-6\gamma$ stoichiometry, where the α -subunits would occupy the core of the molecule, the β -subunits would be localized at the periphery of the α 4-core and the γ -subunits would be located at the exterior of the α 4/ β 4-complex (Tanneberger, et al., 2007). However, this model requires confirmation from structural data.

Three-dimensional (3D) structural studies using electron microscopy techniques carried out on different yeast phosphofructokinases at different states of the catalytic cycle have

provided insights into the structural/functional mechanism of the enzyme. The initial 3D study resolved for the first time the oligomeric architecture of *S. cerevisiae* Pfk in the presence of F6P (Ruiz, et al., 2001). A continuing study by cryoelectron microscopy to 10.8Å resolution permitted to infer the position of the N-terminal domains of the α - and the β subunits and the localization of the catalytic binding region (Ruiz, et al., 2003). In addition, in combination with molecular replacement, it provided initial phases to determine the X-ray structure of a truncated tetramer in the presence of F6P (Mechin, 2002). The 3D structures of the homo-octameric *S. pombe* Pfk determined in the presence of F6P (active state) and in the presence of ATP (inactive state) revealed a similar organization for both, homo- and hetero-octameric Pfk (Benjamin, et al., 2007). These results in combination with the 3D structure of *S. cerevisiae* Pfk in the inactive state, calculated by cryoelectron microscopy (Barcena, et al., 2007), have manifested a strong correlation between structural conformation and kinetic behavior. The structural studies have shown that minor rearrangements of the subunits influence the rotation between the tetramers in the complex, a parameter strongly correlated with the kinetic behavior of the enzyme.

We have carried out 3D reconstructions of *P. pastoris* Pfk from stain-embedded preparations of the enzyme. Random conical techniques and tomography have been crucial to discern between structural instabilities and different orientations of the enzyme in different preparations. After extensive classification of a random conical data set of a homogeneous preparation of *P. pastoris* Pfk, a 3D reconstruction was calculated for each class. The final 3D structure of *P. pastoris* Pfk to 2.5 nm resolution resembles its yeast counterparts. However, additional densities observed in the structure have enabled us to determine the number and positions of the γ -subunits in the complex. We have confirmed the proposed interaction between the γ - and the β -subunits. In addition, we have visualized interactions between the γ - and the α -subunits. The γ -subunit seems to form a bridge between the α - and β -subunits belonging to the two different α/β -dimers within a α_2/β_2 tetramer. These interactions might lock the subunits in a conformation close to the inactive state of other species and might be at the origin of the higher sensitivity to ATP inhibition shown by the *P. pastoris* Pfk.

Materials and Methods

Enzyme purification

Phosphofructokinase from *P. pastoris* was purified from the strain 70382 (DSMZ, Braunschweig, Germany) by using fractionated ammonium sulfate precipitation, interfacial salting out, ion exchange chromatography, size exclusion chromatography and as main step a pseudobiospecific dye-liganded affinity chromatography on Procion Blue H5R-Sepharose (Kirchberger et al. 2002). The purified enzyme was stored in 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM phenylmethanesulphonylfluoride, 5 mM mercaptoethanol, 30% glycerol, pH 7.2, containing additionally 5 mM ATP or 6 mM F6P, at -20°C .

Electron microscopy

A small aliquot of the enzyme suspension was applied to a micro-Bio-Spin P30 column (Bio-Rad) equilibrated with 50 mM sodium phosphate buffer (pH 7.2), supplemented with either 3 mM F6P (active state) or 1 mM ATP and 3 mM MgSO_4 (inactive state). The enzyme was diluted to 30 $\mu\text{g}/\text{ml}$ in the presence of 30 $\mu\text{g}/\text{ml}$ of Tobacco Mosaic Virus (TMV), applied to carbon coated grids and deep stained with 1% uranyl acetate (Ruiz and Radermacher, 2006; Benjamin, et al., 2007). 5 μl of sample were adsorbed onto the grid, after ~ 45 s, the grid was rinsed sample down on three drops (~ 200 μl) of stain by placing the grid sequentially on top of each drop and moving it gently over the surface. After ~ 30 s

in the last drop, the grid was picked up, the excess liquid was wicked off with a filter paper and fast air dried to obtain a deep layer of stain.

The grids were observed on a Tecnai T12 electron microscope (FEI, Holland) equipped with a LaB₆ cathode (Kimball, USA), and a Dual-Axis Tomography Holder (Fischione, USA). Images were recorded at an accelerating voltage of 100 kV, and 52,000 $\times$ nominal magnification under low dose conditions (1000 e⁻/nm²) on S0-163 Kodak film. Random conical data sets were collected with nominal tilt angles of either +70° or -70°. The tilted images were collected with defoci of ~ 2 μ m, while a defocus of ~ 1.5 μ m was used for the untilted or 0° images. Tomography tilt series were collected at a nominal magnification of 42000 $\times$ in a $\pm$ 60° angular range at 4° intervals on a 2048 $\times$ 2048 CCD camera (TVIPS, Germany) using the TVIPS tomography program.

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 7 μ m raster size on an Intergraph SCAI flatbed scanner (Z/I Imaging Corporation, Huntsville, AL) and 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 WEB. Selected particles were windowed with dimensions of 140 $\times$ 140 pixels. The images were contrast normalized using the average density within a ring surrounding the windowed particle.

The analysis of the random conical data set essentially followed the procedure described in Benjamin et al. (2007). The contrast transfer function (CTF) of the microscope was corrected by phase flipping, using a smooth function (Radermacher, et al., 2001). The alignments of the 0° images were carried out using simultaneous translational/rotational alignment techniques based on the cross-correlation of Radon transforms (Radermacher, 1994; Radermacher, 1997). A first reference image, to which the complete image series was aligned, was created by reference-free alignment (Marco, et al., 1996). The aligned images were separated into groups of similar particles by an artificial neural network using XMIPP and the average images of each group were used for multi-reference alignment. Data sets underwent further processing by iterating multireference alignment, and correspondence analysis (van Heel and Frank, 1981; Frank and van Heel, 1982; Bretaudiere and Frank, 1986; Marco, et al., 1996), followed by classification with Diday's method of moving centers (Diday, 1971) and hierarchical ascendant classification (HAC) (command *CL CLA* in SPIDER).

After the final alignment of the 0° images, all of the angles of the tilt images are known. These include the azimuthal angle from alignment of the 0° images and the tilt angle, accurately calibrated during the particle picking step (using the command "*Tilted particles*" in Web). 3D reconstructions were calculated from the tilted images of each class using fast Radon inversion algorithms (Radermacher, 1997; Lanzavecchia, et al., 1999). The alignment of the tilt images was refined using cross-correlations of 2D and 3D Radon transforms (Radermacher, 1994). The refinement was iterated until the resolution of the volume was stable and minimal changes in translation and orientation of the projections were found. Sufficiently strong low-pass filters were employed in each step to prevent over-fitting. Reconstructions that showed only differences in orientation were merged (Ruiz, et al., 2001; Barcena, et al., 2007). After merging, the translational and rotational position of the projections was refined. Volumes were visualized using Chimera (Pettersen, et al., 2004).

Processing of the tomography data was carried out in Spider (version 5.0, with modifications) using a procedure written in the laboratory. The projections of the tilt-series

were aligned to a common origin using cross-correlation algorithms (Guckenberger, 1982; Frank and McEwen, 1992). Starting from the 0° image in a single-axis tilt-series, images were aligned consecutively in two branches, towards the maximum positive and maximum negative tilt angle. Each two images in the series were first stretched by $1/\cos(\alpha)$ perpendicular to the tilt axis, where α is the tilt angle of each projection, and then cross-correlated. The aligned projections were first r^* weighted and tomograms were calculated using back-projection algorithms (Gilbert, 1972; Goitein, 1972; Radermacher, 1992).

Results

3D reconstructions of *P. pastoris* phosphofructokinase (*PpPfk*) were carried out from deep stained preparations of the enzyme in different states. Single particles tilting techniques (random conical and tomography) were used to ascertain the stability and homogeneity of the preparations. Initial studies were performed in the presence of F6P (active state) since this state showed the highest degree of structural stability in Pfk's from other species studied in the laboratory.

The analysis and classification of the 0° images of the random conical data set manifested a large number of views in a (5 × 5) self-organizing map (Fig. 1A). In this map, images (nodes) close together represent small structural differences, while nodes that are far apart show the largest differences. Only the four nodes on the top/right quadrant of the map seem to represent a complete *PpPfk* molecule viewed parallel to its long axis (side-view); the whole molecule shows similar density. The 2D nature of the data did not allow for an unambiguous interpretation of the other nodes on the map. The bottom/left quadrant could represent either the whole molecule viewed perpendicular to its long axis (top-view) or only the bottom half of the enzyme (half-enzyme). The rest of the nodes on the self-organizing map can be interpreted as different orientations (long axis of the molecule at an angle in between 0°–90°) or as partially degraded *PpPfk*s. Random conical 3D reconstructions of the particles belonging to each node revealed that the top/left nodes were half-enzymes and the rest were partially degraded molecules (data not shown).

Similar results were obtained using electron tomography. Figure 1B shows the central section of a characteristic tomogram and the aligned tilt-series and the reconstructed volume are shown as supplementary figures 1 and 2. Particles are found at different heights in the reconstructed tomogram (Suppl. Fig. 2), which indicates that the deep staining method produces well-embedded preparations of the enzyme. In addition, molecules are not visible either at the air/water or the carbon/water interfaces. In the tomogram, the molecules that show smaller radii (similar to the bottom/left quadrant nodes in figure 1A) represent only half-enzymes and not top-views of *PpPfk*. In the presence of F6P, the structure of *PpPfk* seems to be very unstable and the enzyme can easily dissociate into smaller oligomers. Attempts to solve the structure using only the particles that seemed to represent the whole enzymatic complex (~5–10%) would have required an excessive amount of data. Thus, different preparations obtained by slight modifications of the purification and storage protocol were analyzed using random conical techniques (data not shown) to determine the most suitable for structural analysis. These studies revealed the important role played by ATP in the structural stability of the enzyme.

Preparations of *PpPfk* purified in the presence of ATP at all possible steps in the purification and stored in the presence of 5 mM ATP were the best suited for structural studies. The 3D structure of *PpPfk* was analyzed in the presence of ATP instead of F6P to enhance the homogeneity and stability of the enzymatic preparations. In addition, an electron microscopy preparation protocol was adopted to minimize the time in which the enzyme concentration

drops below 0.5 mg/ml to 10–15 minutes. Samples were diluted to the necessary 20–40 $\mu\text{g}/\text{ml}$ for electron microscopy just prior to adsorption onto the grid.

A random conical data set, consisting of 39 tilt-pairs, was collected from deep stained preparations of *PpPfk* in the presence of ATP. A representative tilt-pair is shown in figure 2. The *PpPfk* preparations look similar to those from *S. cerevisiae* Pfk (*ScPfk*) and *S. pombe* Pfk (*SpPfk*) (Ruiz, et al., 2001; Benjamin, et al., 2007). The molecules are oriented with the long axis parallel to the carbon support and adopt different orientations around the long axis. Only a very small fraction of molecules show the top-view (Fig. 2A). In addition, half enzyme molecules like those seen in the bottom/left quadrant of figure 1A are not observed in this preparation. The (9 $\times$ 9) self-organizing map of this data set (Fig. 3) shows striking differences compared to the map of our initial study (Fig. 1A). All the nodes show well-defined density features, with comparable density along the length of the molecule. The molecule shows two distinct domains connected by two thin densities (central connectors). The connectors are either parallel or almost parallel to each other. However, there are no nodes in which the connectors adopt the triangular conformation observed in the *S. pombe* and *S. cerevisiae* enzymes under the same conditions (Barcena, et al., 2007; Benjamin, et al., 2007).

Nine distinctive node average images (Fig. 3, black boxes) were selected as initial references for the classification procedure using a combination of multireference alignment, correspondence analysis and hierarchical ascendant classification. Examples of factor maps from the last round of correspondence analysis are shown in supplementary figure 3. Factor 1 represents the molecule with the long axis inclined at different angles to the carbon support (Suppl. Fig. 3C, horizontal) and factor 2 represents the molecule at different azimuthal angles around its long axis (Suppl. Fig. 3C, vertical). After the fourth round of hierarchical ascendant classification, the data set was divided into 10 different classes (Fig. 4). Class 10 showed the highest membership number (920 molecules), while class 4 only contains 187 molecules (< 4% of the total number of particles in the data set). Class 4 appears to contain a diverse array of views as evidenced by the smoothness of the densities when compared to classes of similar membership numbers (e.g. class 3).

Volumes were calculated from each of the classes. All the classes, except class 4, represented similar structures viewed at different orientations. The molecules seem to lie mainly with the long axis parallel or at a small angle to the carbon support and adopt different azimuthal orientations. After translational and angular refinement of the projections within each class, the volumes were aligned and merged (except class 4). The merged reconstruction containing approximately 5000 particles was refined to a resolution of 2.5 nm using the 0.3 FSC criterion (Fig. 5, (Rosenthal and Henderson, 2003)).

The *PpPfk* is 24.5 nm long and 14.5 nm wide and appears to have a similar structure as the *SpPfk* and *ScPfk*. In the latter case, the enzyme was described as a dimer of tetramers. In the case of *PpPfk*, the molecule is composed of a dimer of hexamers. The two halves of the molecule are related by a combination of a 2-fold symmetry through an axis perpendicular to the long axis of the molecule and an azimuthal rotation. The azimuthal rotation is approximately 50°. In the reconstruction we can also distinguish the characteristic L-shape of the α - and β -subunits forming the catalytic interface. This is more obvious when comparing the Pfk structures from the three different yeasts (Fig. 6). Figure 6A shows the catalytic surface on the top half of the molecules, with the catalytic sites localized at the interface between the α - and the β -subunits (Fig. 6, α -subunit in red, β -subunits in blue). The N-terminal domain of the β -subunits of *PpPfk* and *ScPfk*, and of the external α -subunit of *SpPfk* are labeled with (x), while the N-terminal domain of the α -subunits, which form the central core of the molecule, is labeled with (•). We observed additional densities (Fig. 6,

arrows, γ -subunit in yellow) in the *PpPfk* that do not exist on the other two yeasts. These four additional densities connect the N-terminal domains of the α - and β -subunits from different $\alpha\beta$ -dimers within each $\alpha_2\beta_2$ -tetramer. The diameter of each additional density is about 4.3 nm, which agrees well with the calculated diameter of a globular subunit of about 40 kDa molecular mass. Since *PpPfk* is composed of either four or six γ -subunits of 41 kDa molecular mass in addition to the 4 α - and 4 β -subunits (Tanneberger, et al., 2007), we can conclude that the four additional densities in the structure correspond to four novel γ -subunits. The apparent degree of the interactions between the α - and γ -subunits and β - and γ -subunits is comparable and much larger than the interaction between the α - and the β -subunits at the catalytic interface, as was seen by increasing the density threshold for visualization (data not shown).

Discussion

We have addressed the structural characterization of *PpPfk* by electron microscopy studies to determine the quaternary structure of the enzyme, and the number and locations of the γ -subunits, unique to this family of yeasts. Biochemical and genetic studies have shown that the γ -subunits are integral constituents of the *PpPfk* and are probably present in, at least, equimolar ratio with the α - and β -subunits. In addition, cross-linking and limited proteolytic degradation data have led to the hypothesis that the quaternary structure of the *PpPfk* is similar to the structure of the *ScPfk* (Tanneberger, et al., 2007). The 4 α -subunits would form the core, the 4 β -subunits would be bound on the outside and the γ -subunits would be localized at the exterior of the $\alpha_4\beta_4$ complex, bound to the β -subunits.

Random conical and electron tomography techniques have proven instrumental for assessing the suitability of *PpPfk* samples for structural studies. The first 3D electron microscopy analysis of *PpPfk* in the presence of F6P (active state) evidenced that *PpPfk* preparations were not homogeneous (Fig. 1, Suppl. Figs. 1 and 2). The 3D reconstructions of the different classes showed that the enzyme either dissociated into halves, lost some of the external subunits (β -subunits), or even whole dimers ($\alpha\beta$ -dimers). This effect could not be visualized by gel electrophoresis, since all the subunits seemed to be present with the correct stoichiometry and had not suffered proteolysis (data not shown). Furthermore, a 2D analysis of the images was not able to reveal the nature of the variability due to the overlapping features in 2D projections. Images showing small molecules could have been interpreted as top-views of the molecule, which would have had a deleterious effect in the final 3D reconstruction, since they represented only half-molecules. Only 3D tilting techniques, such as random conical tilt, are able to discriminate between different orientations and different conformations of a molecule, thus providing the tool to control and quantify the stability and homogeneity of the preparations and ensuring the validity of the 3D reconstruction.

The 3D reconstructions of *PpPfk* in the presence of ATP show that the quaternary structure is, in principle, similar to the *ScPfk* and the *SpPfk*. The molecule has a cylindrical or ellipsoidal shape with its long axis approximately one and a half times longer than the long axis (Table 1). The length of the *PpPfk* (24.5 nm) is identical to that of the *ScPfk* and is more than 15% longer than the *SpPfk*, and the apical ends of *PpPfk* are similar to those of *ScPfk* and much better defined than in *SpPfk*. These observations are interesting since both *PpPfk* and *SpPfk* structures were obtained from deep-stained preparations and the *ScPfk* from cryo-preparations and appear to indicate that some features are better preserved in *PpPfk* than in *SpPfk*. The largest width (perpendicular to the catalytic surface, Fig. 6B, bottom-half of the molecule) of *PpPfk* (14.5 nm) is similar to that of *SpPfk*, but both are about 10% smaller than the *ScPfk*. However, the smallest width (parallel to the catalytic surface, Fig. 6A, top-half of the molecule) of *PpPfk* is about 40% smaller than the *ScPfk* (12.5 nm), which indicates that the molecule is very sensitive to flattening forces. The

catalytic surface view (Fig. 6A, top-half of the molecule) displays the characteristic L-shaped subunits of yeasts Pfk (Fig. 6, α -subunit in red, β -subunit in blue). The horizontal arms of the L-shaped *PpPfk* subunits, showing a length of 8 nm as in the *ScPfk* subunits, correspond to the N-terminal domains of the α - and β -subunits and interact in an antiparallel fashion forming two of the eight catalytic sites of the enzyme (Ruiz, et al., 2001; Ruiz, et al., 2003). At the distal-ends of the *PpPfk* molecule, the tips of the L-shaped subunits (β -subunits, Fig. 6, blue) are easily seen lifting outwards like in the *ScPfk* (Barcena, et al., 2007). The tip of the β -subunit is a possible binding site for the γ -subunit. Since a γ -subunit would be bound to each β -subunit, a density corresponding to a molecular mass of 82 kDa should be visualized in this area. The similarities observed in projection averages of the wild type *PpPfk* and a mutant *PpPfk* lacking the γ -subunit in this apical region (Suppl. Fig. 4) provide additional support to rule out the tip as the site binding site for the γ -subunit.

We have observed four additional densities in the *PpPfk* (Fig. 6, arrows, γ -subunit in yellow), which are bridging the N-terminal domains of the α - ($\bullet$) and β -subunits ($\times$). These densities resemble linguiform lobes that extend outwards at about 40° and point towards the horizontal mid-section of the molecule. Since *PpPfk* has a minimum of four copies of a novel γ -subunit (Tanneberger, et al., 2007) and the estimated molecular mass of each of these densities is ~ 40 kDa, we have identified these additional densities with four γ -subunits. Our reconstructions display interactions of commensurable density between the γ -subunit and both the α - and β -subunits. Close interactions between the γ - and β -subunits have been detected using cross-linking experiments with purified *PpPfk* followed by gel electrophoresis and sequencing of well separated bands with molecular masses larger than 100 kDa (Tanneberger, et al., 2007). However, additional interactions might have remained undetected due to the resolving power of the procedure used. Limited proteolysis experiments performed on the purified *PpPfk* in the presence of ATP, using chymotrypsin, revealed that only the γ - and β -subunits were truncated (Tanneberger, et al., 2007). Similar experiments carried out on *ScPfk* showed a 200 amino acids truncation of the N-terminal domains of both the α - and the β -subunits and an additional truncation of 80 amino acids from the C-terminus of the α -subunit (Kopperschlager, et al., 1993). The interaction between the α - and γ -subunits, observed in the 3D reconstruction, could be protecting the α -subunit from proteolysis in two ways. In one way, the γ -subunit could be interacting with the specific residues of the α -subunit involved in proteolysis, thus shielding putative cleavage sites and making them unavailable to the protease. In another, the γ -subunit could have induced a conformational change on the α -subunit, upon binding, which decreases the affinity of the protease.

The bridges formed by the γ -subunits *PpPfk* have created a molecule that resembles a cage, with a very open structure. These interactions have introduced additional stress that can be easily transmitted between the $\alpha\beta$ -dimers within each half of the molecule that did not exist in the *ScPfk* and *SpPfk*. The open cage-like structure of *PpPfk* makes the molecule more sensitive than its yeast's counterparts to flattening forces experienced by samples during electron microscopy preparations. Since the molecules are well stain-embedded (see above, Suppl. Fig. 2) interactions with either the air/water or the carbon/water interfaces can be discarded as the cause for the observed flattening. In addition, the molecules are always flattened in the same direction with respect to the molecule geometry, independent of their orientation relative to the carbon support film. The causative agent for the flattening effect might have to be searched among other physical forces exerted on the molecules upon drying, which might be very large at the molecular scale. Interestingly, these compression forces appear to have negligible effects on small structural features if the molecules are well stain-embedded. Detailed architectural features inferred from flattened molecules corresponded well to the features of well-preserved molecules in Pfk from other species

(Ruiz, et al., 2001; Ruiz, et al., 2003; Benjamin, et al., 2007) and also in the Ca^{2+} -release channel (Wagenknecht, et al., 1989; Radermacher, et al., 1994).

The number and locations of the γ -subunit in the 3D structure of *PpPfk* can lead to an explanation for the kinetic properties of this enzyme (Kirchberger, et al., 2002; Tanneberger, et al., 2007). The inhibition of *PpPfk* by ATP is stronger than in other yeast Pfk, and the activation by F26bP is significantly lower. However, a γ -subunit null-mutant shows a lesser inhibition by ATP, which led to the hypothesis that the γ -subunit is involved in fine tuning the enzyme kinetics and it is important for the adaptation of *P. pastoris* to changes in the energy resources. In prior 3D studies of *ScPfk* and *SpPfk*, a correlation between the enzyme kinetics and structural conformation was established (Ruiz, et al., 2003; Barcena, et al., 2007; Benjamin, et al., 2007). The azimuthal rotation between the tetramers was larger for the active state (65° – 75°) than for the inactive state (46° – 50°). The angle between the top- and bottom-halves of the *PpPfk* (50°) is similar to the angle observed for other Pfk in the inactive state. In addition, it was shown that this change in the azimuthal rotation between the tetramers was caused by a sliding outward of the N-terminal domains of the α - and β -subunits within the $\alpha\beta$ -dimer, which precluded the interaction between F6P and the ATP γ -phosphate (Barcena, et al., 2007). The bridge between the N-terminal domains of the α - and β -subunits of two different $\alpha\beta$ -dimers within one half of the molecule can lock the subunits in the inactive state in *PpPfk*. The activators would also have to overcome this blockage enforced by the γ -subunit.

In summary, we have used 3D electron microscopy tilting techniques (random conical and tomography) to fine-tune and select the best purification and storage method to obtain the most stable and homogeneous preparation of *PpPfk* for structural studies. We have calculated a final 3D reconstruction of *PpPfk* in the presence of ATP. Four γ -subunits were identified, which show interactions not only with the N-terminal domain of the β -subunits, but also with the N-terminal domains of the α -subunits. Our current 3D model of the *PpPfk* provides clues to better interpret the biochemical and kinetic data for this enzyme and bring us closer to understanding the structural/functional relationship of the eukaryotic phosphofructokinase family.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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]
- Barcena M, Radermacher M, Bar J, Kopperschlager G, Ruiz T. The structure of the ATP-bound state of *S. cerevisiae* phosphofructokinase determined by cryo-electron microscopy. *J Struct Biol*. 2007; 159:135–43. [PubMed: 17493831]
- Benjamin S, Radermacher M, Bar J, Edelmann A, Ruiz T. Structures of *S. pombe* phosphofructokinase in the F6P-bound and ATP-bound states. *J Struct Biol*. 2007; 159:498–506. [PubMed: 17643314]
- 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, Bar J. Molecular genetics of 6-phosphofructokinase in *Pichia pastoris*. *Yeast.* 2002; 19:949–56. [PubMed: 12125051]
- Flores CL, Martinez-Costa OH, Sanchez V, Gancedo C, Aragon JJ. The dimorphic yeast *Yarrowia lipolytica* possesses an atypical phosphofructokinase: characterization of the enzyme and its encoding gene. *Microbiology.* 2005; 151:1465–74. [PubMed: 15870456]
- Frank, J.; McEwen, B. Alignment by Cross-Correlation. In: JF, editor. *Electron Tomography*. Plenum Press; 1992. p. 205–214.
- 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]
- Gilbert PF. The reconstruction of a three-dimensional structure from projections and its application to electron microscopy. II. Direct methods. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 1972; 182:89–102.
- Goitein M. Three-dimensional density reconstruction from a series of two-dimensional projections. *Nuclear Instruments and Methods.* 1972; 101:509–518.
- Guckenberger R. Determination of a common origin in the micrographs of tilt series in three-dimensional electron microscopy. *Ultramicroscopy.* 1982; 9:167–174.
- Heinisch J, Ritzel RG, von Borstel RC, Aguileria A, Rodicio R, Zimmermann FK. The phosphofructokinase genes of yeast evolved from two duplication events. *Gene.* 1989; 78:309–21. [PubMed: 2528496]
- Heinrich R, Rapoport SM, Rapoport TA. Metabolic regulation and mathematical models. *Progress in Biophysics & Molecular Biology.* 1977; 32:1–82. [PubMed: 343173]
- Hofmann E. The significance of phosphofructokinase to the regulation of carbohydrate metabolism. *Reviews of Physiology Biochemistry & Pharmacology.* 1976; 75:1–68.
- Kirchberger J, Bar J, Schellenberger W, Dihazi H, Kopperschlager G. 6-phosphofructokinase from *Pichia pastoris*: purification, kinetic and molecular characterization of the enzyme. *Yeast.* 2002; 19:933–47. [PubMed: 12125050]
- 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]
- Lanzavecchia S, Bellon PL, 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]
- 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.
- 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]

- Poorman RA, Randolph A, Kemp RG, Henrikson RL. Evolution of phosphofructokinase--gene duplication and creation of new effector sites. *Nature*. 1984; 309:467–9. [PubMed: 6233492]
- Radermacher, M. Weighted Back-Projection Methods. In: Frank, J., editor. *Electron Tomography*. Plenum Press; 1992. p. 91-116.
- 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, Rao V, Grassucci R, Frank J, Timmerman AP, Fleischer S, Wagenknecht T. Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. *J Cell Biol*. 1994; 127:411–23. [PubMed: 7929585]
- Radermacher M, Ruiz T, Wiczorek 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]
- 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. *J Struct Biol*. 2001; 136:167–80. [PubMed: 12051897]
- Ruiz T, Mechlin I, Bar 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]
- 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]
- Tanneberger K, Kirchberger J, Bar J, Schellenberger W, Rothemund S, Kamprad M, Otto H, Schoneberg T, Edelmann A. A novel form of 6-phosphofructokinase. Identification and functional relevance of a third type of subunit in *Pichia pastoris*. *J Biol Chem*. 2007; 282:23687–97. [PubMed: 17522059]
- Tijane MN, Seydoux FJ, Hill M, Roucoux C, Laurent M. Octameric structure of yeast phosphofructokinase as determined by crosslinking with disuccinimidyl beta-hydroxybenzoate. *FEBS Letters*. 1979; 105:249–53. [PubMed: 158542]
- van Heel M, Frank J. Use of multivariate statistics in analysing the images of biological macromolecules. *Ultramicroscopy*. 1981; 6:187–94. [PubMed: 7268930]
- Wagenknecht T, Grassucci R, Frank J, Saito A, Inui M, Fleischer S. Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. *Nature*. 1989; 338:167–70. [PubMed: 2537473]
- Yuan W, Tuttle DL, Shi YJ, Ralph GS, Dunn WA Jr. Glucose-induced microautophagy in *Pichia pastoris* requires the alpha-subunit of phosphofructokinase. *J Cell Sci*. 1997; 110:1935–45. [PubMed: 9296392]

Figure 1.

A) Self-organizing map of 0° images from a random conical data set at 0° tilt of early *P. pastoris* Pfk preparations in the presence of F6P. The numbers indicate the class membership. Scale bar, 10 nm. B) Central slice from a tomographic reconstruction of *P. pastoris* Pfk preparations in the presence of F6P. Scale bar, 100 nm.

Figure 2.
A and B, Tilt pair of *P. pastoris* Pfk preparations in the presence of ATP; 0° micrograph (A) and tilted micrograph (70°) (B). C and D, Extracted images from the 0° micrograph (C) and tilted micrograph (D). Scale bar: 100 nm.

Figure 3. Self-organizing map of 0° images from a random conical data set at 0° tilt of *P. pastoris* Pfk preparations in the presence of ATP. The numbers indicate the class membership. The Black boxes show the classes that were used for the initial multireference alignment step.

Figure 4.

Class averages from the last classification step. Indicated is the class number (top) and the number of images included in the class (bottom). Scale bar: 10 nm.

Figure 5.

Left) Gallery of surface views in 30 ° steps of the 3D reconstruction of *P. pastoris* Pfk. Scale bar, 10 nm. Right) Fourier shell correlation curve (solid line) and 5 times noise correlation curve (dashed line).

Figure 6.

Surface representation of the 3D reconstruction Pfk from stain embedded preparations of *P. pastoris* (*Pp*, left column), cryo preparations of *S. cerevisiae* (*Sc*, center column) and stained embedded preparations of *S. pombe* (*Sp*, right column). A) “front” view, showing the catalytic surface and B) “side” view. (•) indicates the N-terminal domain of the α -subunits (colored in red) and (x) the N-terminal domain of the β -subunits of *P. pastoris* and *ScPfk*s and the N-terminal domain of the external α -subunit of *S. pombe* (all colored in blue), the γ -subunits of *P. pastoris* are colored in yellow. Surfaces have been manually colored aided by the visualization of surfaces at different thresholds. Scale bar. 10 nm.

Table 1

Molecular weights and dimensions of Pfk from different species calculated from the 3D reconstruction of the enzyme in the presence of ATP. *ScPfk* (*S. cerevisiae* Pfk), *PpPfk* (*P. pastoris* Pfk), *SpPfk* (*S. pombe* Pfk).

	<i>ScPfk</i>	<i>PpPfk</i>	<i>SpPfk</i>
<i>Molecular mass (kDa)</i>	835 ± 32	975 ± 28	790 ± 30
<i>Length (nm)</i>	24.5	24.5	21.0
<i>Large width (nm)</i>	16.2	14.5	14.5
<i>Small width (nm)</i>	12.5	9.0	10.6
<i>Rotation angle</i>	46°	50°	50°