Structure and Activity of the Metal-independent Fructose-1,6-bisphosphatase YK23 from Saccharomyces cerevisiae

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Fructose-1,6-bisphosphatase (FBPase), a key enzyme of gluconeogenesis and photosynthetic CO$_2$ fixation, catalyzes the hydrolysis of fructose 1,6-bisphosphate (FBP) to produce fructose 6-phosphate, an important precursor in various biosynthetic pathways. All known FBPases are metal-dependent enzymes, which are classified into five different classes based on their amino acid sequences. Eukaryotes are known to contain only the type-I FBPases, whereas all five types exist in various combinations in prokaryotes. Here we demonstrate that the uncharacterized protein YK23 from Saccharomyces cerevisiae efficiently hydrolyzes FBP in a metal-independent reaction. YK23 is a member of the histidine phosphatase (phosphoglyceromutase) superfamily with homologues found in all organisms. The crystal structure of the YK23 apo-form was solved at 1.75-Å resolution and revealed the core domain with the α/β/α-fold covered by two small cap domains. Two liganded structures of this protein show the presence of two phosphate molecules (an inhibitor) or FBP (a substrate) bound to the active site. FBP is bound in its linear, open conformation with the cleavable C1-phosphate positioned deep in the active site. Alanine replacement mutagenesis of YK23 identified six conserved residues absolutely required for activity and suggested that His$_{13}^\text{in}$ and Glu$_{18}^\text{in}$ are the primary catalytic residues. Thus, YK23 represents the first family of metal-independent FBPases and a second FBPase family in eukaryotes.

Fructose-1,6-bisphosphatase (FBPase) catalyzes the removal of phosphate 1 from fructose 1,6-bisphosphate to produce fructose 6-phosphate, an important precursor in various biosynthetic pathways. FBPase is a key, rate-controlling enzyme of gluconeogenesis, an important metabolic pathway that allows the cells to synthesize glucose and grow on non-carbohydrate carbon sources, such as glycerol, organic acids, and amino acids. Gluconeogenesis is also responsible for excessive glucose production found in type 2 diabetes, which is a growing worldwide health concern. Because FBPases represent the major control point and function only in gluconeogenesis in mammals, they are recognized as an attractive target for the development of drugs for the treatment of type 2 diabetes. FBPase also functions at the branch point between the regenerative phase of the photosynthetic CO$_2$ fixation cycle (Calvin), which is the primary pathway of carbon fixation, and the starch biosynthesis (4). In addition, FBPase is required for virulence in Mycobacterium tuberculosis and Leishmania major and plays an important role in the production of lysine and glutamate by Corynebacterium glutamicum (5, 6).

Most characterized FBPases belong to the superfamily of lithium-sensitive metal-dependent phosphatases that also includes three families of inositol phosphatases (clan CL0171, Pfam data base) (7). Based on the amino acid sequence, FBPases can be assigned to one of the five proposed classes (8–10). All known FBPases require a divalent metal cation for activity (Mg$^{2+}$ or Mn$^{2+}$), are inhibited by Li$^+$, and their activity is often regulated by AMP, fructose 2,6-bisphosphate, and phosphoenolpyruvate (11–13). Types I, II, and IV FBPases and inositol monophosphatases have similar three-dimensional structures with a sugar phosphatase-fold (αβαβα) suggesting a common evolutionary origin and catalytic mechanism (14–16). All five types of FBPases are normally found in prokaryotes, where types I, II, and III are more common to bacteria (8), type IV are mostly found in archaea (10), and type V in thermophiles from both domains (9). The majority of organisms have more than one FBPase, mostly the combination of types I and II or I and III (8). For example, Escherichia coli has three FBPases (one type I and two type II), which are differentially expressed and regulated and, therefore, are proposed to play complementary roles.
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roles (8, 16). In the Arabidopsis thaliana genome, there are four genes encoding the type I FBPs: the cytosolic FBPase (341 amino acids, P25851, 50% sequence identity to cytosolic FBPase), the FBPase-like protein (404 amino acids, Q9FMF1, 42% sequence identity), and the sedoheptulose-1,7-bisphosphatase (SBPase) (393 amino acids, P46283 or Q940F8, 29% sequence identity).

Like FBP, sedoheptulose 1,7-bisphosphatase (SBP), a seven-carbon phosphorylated carbohydrate, is a metabolic intermediate of the regenerative phase of the photosynthetic carbon reduction (Calvin) cycle (17). The experimentally characterized plant SBPases also show significant activity against FBP (10–20% of the activity with SBP) (BRENDA data base).

In cyanobacteria (which have two FBPases, types I and II) the type II FBPases (GlpX-like) have been shown to have similar activities against FBP and SBP and are annotated as a FBPase/SBPase (18). The comparison of sequences and biochemical properties of FBPases and SBPases suggested that these enzymes are related both functionally and evolutionarily (19).

In Synechocystis sp. PCC 6803 (the only known FBPase, and its function is allosterically controlled by AMP and fructose 2,6-bisphosphate, as well as by ubiquitin-based proteolytic degradation to avoid futile cycling with phosphofructokinase (8, 20–23). However, with more than 1,000 uncharacterized genes in the yeast genome (24), experimental characterization will likely reveal novel enzymes. For example, most of the over 25 predicted histidine phosphatases (HP) and haloacid dehalogenase-like hydrolases remain uncharacterized and represent a potential source of novel phosphatases including FBPs.

In this work we recombinitely expressed and purified two uncharacterized members of the HP superfamily from S. cerevisiae, YK23 and YO283 in E. coli. We demonstrated that YK23 is a novel FBPase, whereas YO283 is a nonspecific, broad substrate range phosphatase. YK23 was characterized biochemically and its crystal structure was solved in the apo-form and in complex with phosphate or FBP. These structures together with the mutational analysis of YK23 provide insight into the molecular mechanisms of substrate selectivity and catalysis of the first metal-independent FBPase.

EXPERIMENTAL PROCEDURES

Gene Cloning, Overexpression, and Protein Purification—The genes encoding YK23 (YKR043C) and YO283 (YOR283W) were amplified by PCR using S. cerevisiae genomic DNA. The amplified fragments were cloned into a modified pET15b vector (Novagen) using the In-Fusion Dry-Down PCR cloning kit (Clontech) as described by the manufacturer. The modified pET15b vector contains an N-terminal His tag followed by a tobacco etch virus protease cleavage site (ENLYFQG). The overexpression plasmids were transformed into the E. coli BL21(DE3) Gold strain (Stratagene). YK23 and YO283 were purified using immobilized metal ion affinity chromatography on nickel affinity resin (Qiagen) with high yield (>100 mg/liter of culture) and homogeneity as described previously (25). Purified proteins were frozen in liquid nitrogen and stored at −80°C.

The oligomeric state of YK23 was determined using gel filtration analysis on a Superdex 200 10/300 column (GE Healthcare). The column was equilibrated with 50 mM HEPES-K (pH 7.5) and 250 mM NaCl using an AKTA FPLC (GE Healthcare). Retention time of the native YK23 was used to estimate the relative molecular mass of the protein via linear regression using ribonuclease A (13.7 kDa), ovalbumin (43 kDa), and aldolase (158 kDa) as standards.

Enzymatic Screens and Assays—Purified YK23 and YO283 were initially screened for the presence of several general enzymatic activities including phosphatase, phosphodiesterase, proteases, esterases, dehydrogenase, and oxidase (26). In addition, secondary phosphatase screens with 93 phosphorylated metabolites (supplemental Table S1) were performed to identify the preferred natural substrates for these proteins (26).

Phosphatase activity against FBP and other substrates (fructose 1-phosphate, glyceraldehyde 3-phosphate, and erythrose 4-phosphate) was measured spectrophotometrically using the Malachite Green reagent (27). The reactions were conducted using a mixed buffer system (28) that consisted of 50 mM each of sodium acetate, MES, PIPES, HEPES, and TAPS. Briefly, the reaction mixture (160 µl) contained 50 mM mixed buffer, 0.5 mM substrate, and 0.1–0.25 µg of enzyme. Following a 15-min incubation at 30°C, the reaction was terminated by the addition of 40 µl of Malachite Green reagent (27) and the amount of released Pi was calculated based on absorbance at 630 nm. For the determination of the Km and Vmax, phosphatase activity was determined over a range of substrate concentrations (between 0.2 and 5 mM for FBP and up to 8 mM for Fru-1P, glyceraldehyde 3-phosphate, and erythrose 4-phosphate). Kinetic parameters were calculated by non-linear regression analysis of raw data fit to the Michaelis-Menten function using the GraphPad Prism Software (version 4.00 for Windows, GraphPad Software, San Diego, CA). A mild phosphate detection method was used in assays involving hydrolysis of glyceraldehyde 3-phosphate and erythrose 4-phosphate. The protocol for mild phosphate detection was adapted to microwell plates from Saheki et al. (29).

In this work, we recombinitely expressed and purified two uncharacterized members of the HP superfamily from S. cerevisiae, YK23 and YO283 in E. coli. We demonstrated that YK23 is a novel FBPase, whereas YO283 is a nonspecific, broad substrate range phosphatase. YK23 was characterized biochemically and its crystal structure was solved in the apo-form and in complex with phosphate or FBP. These structures together with the mutational analysis of YK23 provide insight into the molecular mechanisms of substrate selectivity and catalysis of the first metal-independent FBPase.
a decrease in absorbance at 340 nm. A 1-ml reaction mixture contained 80 mM triethanolamine buffer (pH 7.6), 7 mM 3-PGA, 0.7 mM ADP, 1.4 mM diphosphoglycerate, 0.15 mM β-NADH, 2.5 mM MgSO$_4$, 100 mM KCl, 14 units of pyruvate kinase, 20 units of 1-lactic acid dehydrogenase, and 3 units of enolase. Following the addition of YK23, absorbance at 340 nm was monitored for 5 min. E. coli phosphoglycerate mutase (gpmI) was used as a positive control.

**Site-directed Mutagenesis of YK23**—Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis protocol (Stratagene). The amino acids selected for mutagenesis were changed to alanine. The modified pET15b vector containing the wild type YKR043C DNA was used as a template for mutagenesis. The standard PCR mixture contained 50–100 ng of template DNA and 250 ng of each mutagenizing primer. The methylated template plasmid was digested with DpnI, and 2–4 µl of each reaction was used to transform competent E. coli DH5α cells. Plasmids were purified from ampicillin-resistant colonies using the QIAprep Spin mini prep kit (Qiagen) and the presence of mutations was verified by DNA sequencing. Plasmids containing the desired mutations were transformed into E. coli BL21(DE3) cells. Mutated YK23 proteins were overexpressed in E. coli and purified using the same protocol as for the wild type protein.

**Protein Crystallization and Data Collection**—Crystals of the selenomethionine-substituted YK23 were grown at 22 °C using the hanging drop vapor diffusion method. 2 µl of protein sample was mixed with an equal volume of reservoir solution as previously described (30). Prior to set up, purified YK23 (29 mg/ml) was mixed (1/10, v/v) with 1.5 mg/ml of trypsin solution (in 1 mM HCl and 2 mM CaCl$_2$) (31). The multilayered, plate-like crystals appeared after 2 days in the presence of 0.2 M trillithium citrate, pH 8.1, 16% PEG-3350 and 4% (±)-2-methyl-2,4-pentanediol. Diffractioning crystals were flash frozen in liquid nitrogen. Crystals of YK23 with bound phosphates were obtained by crystallization in the presence of 0.1 M trillithium citrate (pH 8.1), 15% PEG-3350 and 8% (±)-2-methyl-2,4-pentanediol. Diffractioning crystals were flash frozen in liquid nitrogen. Crystals of YK23 with bound phosphates were obtained by crystallization in the presence of 0.1 M trillithium citrate (pH 8.1), 15% PEG-3350 and 8% (±)-2-methyl-2,4-pentanediol. Diffractioning crystals were flash frozen in liquid nitrogen.

**Structure Determination**—The apo-structure of YK23 was solved using multiwavelength anomalous dispersion using the selenium anomalous signal. A two-wavelength dataset of the selenomethionine-containing crystal was collected on a Rigaku R-Axis IV+ image plate (Rigaku Americas, TX). The structure was solved by molecular replacement using the coordinates of one monomer of the apo-structure. All four molecules were found using the molecular replacement program PHASER, and this initial model was then modified with cycles of manual building with COOT and restrained refinement as described above with the apo-structure. As with the apo-structure, data collection and refinement statistics are summarized in Table 1.

**RESULTS AND DISCUSSION**

**Enzymatic Screening Revealed Phosphatase Activity in YK23 and YO283**—To identify novel yeast FBPsases, we selected the poorly characterized HP superfamily, also known as the phosphoglycerate mutase superfamily (IPR013078, over 9,000 sequences in the InterPro data base). The first experimentally characterized member of this superfamily was the yeast phosphoglyceromutase GPM1, which interconverts 3-phosphoglyceric acid (3-PGA) and 2-phosphoglyceric acid (2-PGA) in both glycolysis and gluconeogenesis (41). However, it seems that like
the haloacid dehalogenase-like hydrolase superfamily the vast majority of phosphoglycerate mutase superfamily proteins are phosphatases that dephosphorylate various small molecules (fructose 2,6-bisphosphate, glucose 1-phosphate) or proteins (e.g. the HPt domain of ArcB) (42). Therefore, this protein group was recently renamed as the HP superfamily (42). Structurally and biochemically characterized HP family members include yeast phosphoglyceromutase GPM1 (43) and rat fructose-2,6-bisphosphatase (44). The *S. cerevisiae* genome has 12 genes encoding HP proteins, including the biochemically characterized phosphoglyceromutase GPM1 (P00950) and fructose-2,6-bisphosphatase FBP26 (P32604), whereas other proteins are annotated as unknown or predicted phosphoglycerate mutases (supplemental Table S2).

The uncharacterized HP proteins YK23 (YKR043C) and YO283 (YOR283W) from *S. cerevisiae* were cloned and purified as part of a structural proteomics project focused on unknown microbial proteins (SPiT). Both YK23 and YO283 are medium-size soluble proteins (271 and 230 amino acids, respectively) annotated as an uncharacterized protein (YK23) or as a probable phosphoglyceromutase (YO283). Recently, YO283 was shown to have phosphatase activity against 3-PGA, but the substrate preference of this protein was not studied (45). Both YK23 and YO283 show low sequence similarity to each other (26% sequence identity) (Fig. 1) and GPM1 (26 and 28% sequence identity for YK23 and YO283, respectively). Our general enzymatic screens with purified YK23 and YO283 revealed the presence of metal-independent phosphatase activity against the general phosphatase substrate *p*-nitrophenyl phosphate (0.7 and 40 μmol/min mg of protein, respectively).

To identify the potential natural substrates for YK23 and YO283, both proteins were screened against a set of 93 phosphorylated substrates representing the main phosphometabolome groups (nucleotides, carbohydrates, amino acids, organic acids, and cofactors) (supplemental Table S1) (46). In this screen, YK23 showed the highest activity against FBP, whereas YO283 was active against a broad range of phosphorylated substrates including nucleoside tri- and diphosphates, phosphorylated organic acids, and amino acids (Fig. 2, A and B). YO283 also hydrolyzed two phospho-Tyr containing peptides (DADE-pY-LIPQQG and IEDNE-pY-TARQG), but showed no activity against phytic acid, phosphorylated carbohydrates, and nucleoside monophosphates indicating that this protein exhibits some degree of substrate selectivity. Thus, YO283 represents a
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YK23 showed no phosphoglycerase mutase activity with 3-phosphoglycerate as well as no phosphatase activity against glucose 1,6-bisphosphate or fructose 2,6-bisphosphate. The recently characterized TIGAR (TP53-induced glycolysis and apoptosis regulator) phosphatases from human and zebrafish were active against both fructose 2,6-bisphosphate (Fru-2,6-bisP) and FBP, but substrate affinity to Fru-2,6-bisP was much higher (52). As YK23 and TIGAR proteins share low sequence similarity (25% sequence identity), they likely belong to different subfamilies of HP phosphatases. Analysis of the reaction products of FBP hydrolysis by YK23 revealed that like a typical FBPase this enzyme produced fructose 6-phosphate and therefore can be classified as an FBPase.

With all natural substrates, YK23 showed classical hyperbolic saturation kinetics and exhibited the highest activity and substrate affinity toward FBP (Table 1). Some of the known metal-dependent FBPs display sigmoidal saturation kinetics with FBP suggesting positive cooperativity between the subunits in substrate binding, whereas in other enzymes the cooperativity is induced by the FBPase inhibitor fructose 2,6-bisphosphate (Fru-2,6-bisP) and FBP, but substrate affinity to Fru-2,6-bisP was much higher (52). As YK23 and TIGAR proteins share low sequence similarity (25% sequence identity), they likely belong to different subfamilies of HP phosphatases. Analysis of the reaction products of FBP hydrolysis by YK23 revealed that like a typical FBPase this enzyme produced fructose 6-phosphate and therefore can be classified as an FBPase.

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| TABLE 2 |
| Crystallographic data collection and model refinement statistics |
| Values in parentheses are for the highest resolution shell. |

| | YK23 | YK23 | YK23 |
|---|---|---|---|
| | wild type (apo-structure) (PDB 3F3K) | wild type (+ phosphate) (PDB 3LG2) | H13A (+ FBP) (PDB 3L4A) |
| Data collection | | | |
| Space group | a=a=84.0 b=86.3 c=100.2 | a=83.3 b=83.4 c=104.7 | a=83.6 b=84.0 c=101.6 |
| Wavelength (Å) | 0.97948 | 0.97931 | 0.97928 |
| Crystal contacts in the final model using the quaternary structure prediction server, PISA, predicts that the protein is a | 50-1.75(1.78-1.75) | 50-2.50(2.59-2.50) | 50-2.50(2.54-2.50) |
| Kd and Ks (Å) | 0.074(0.265) | 0.078(0.458) | 0.093(0.482) |
| Complete (Å) | 98.2(98.4) | 99.8(98.4) | 91.3(93.3) |
| Redundancy (%) | 9.9(8.9) | 6.1(5.4) | 3.0(2.7) |
| Refinement | | | |
| Resolution (Å) | 1.35-1.75 | 1.35-2.60 | 3.05-3.49 |
| No. reflections | 72653 | 29898 | 21560 |
| Rmerge / Rfree | 14.1/17.0 | 22.0/27.0 | 22.3/26.6 |
| No. atoms | 5349 | 8688 (phosphate) | 8430 |
| Protein | 4374 | 3F3K | |
| Major ligand | 36 | 4 | 57 |
| Other ligands/ solvent | 939 | 29.6 | 24.4 |
| B-factors | 15.7 | 4 | 57 |
| R.m.s. deviations | 0.013 | 0.016 | 0.015 |
| Bond lengths (Å) | 1.35 | 1.65 | 1.56 |
| Bond angles (°) | 91.7 | 88.6 | 88.7 |
| Ramachandran plot | 1.8 | 10.9 | 10.4 |
| Addition allowed (%) | 4.5 | 6.6 | 3.4 |
| Generously allowed (%) | 0.5 | 0.6 | 0.9 |
| Disallowed (%) | 0.0 | 0 | 0 |

1 \[ R_{merge} = \sum \frac{|I_i - \langle I \rangle|}{\sum I_i}, \] where \( I_i \) and \( \langle I \rangle \) are the observed and the calculated structure factors, respectively. \( R_{merge} \) calculated using 5% of total reflections randomly chosen and excluded from the refinement.

2 \[ R_{free} = \frac{\sum_{I_{obs}} - \sum_{I_{calc}}}{\sum_{I_{obs}}}, \] where \( I_{obs} \) and \( I_{calc} \) are the observed and the calculated structure factors, respectively.

tion, both ATP and Pi, inhibited the hydrolysis of FBP by YK23 with IC\textsubscript{50} of 1.25 and 4 mm, respectively. High NaCl concentration (0.2 M) resulted in ~50% reduction of the YK23 FBPase activity and the presence of 50 mm KCl produced a 3-fold decrease in \( K_{m} \) (data not shown). Thus, our biochemical results indicate that YK23 is a novel FBPase that does not require metals for activity and is insensitive to common FBPase inhibitors such as AMP and Li\textsuperscript{+}.

Crystal Structure of YK23 in a Free State—The crystal structure of YK23 was solved to 1.75-Å resolution using selenomethionine-substituted protein and multiple wavelength anomalous dispersion (PDB code 3F3K) (Table 2). Analysis of the crystal contacts in the final model using the quaternary structure prediction server, PISA, predicts that the protein is a homodimer (Fig. 3), consistent with the results of our gel-filtration experiments (61.1–64.7 kDa). The monomer core structure has an HP-fold (α/β/α sandwich) with a six-stranded mixed β-sheet (β1, β2, β3, B4, B8, and B9) flanked by α-helices on both sides (Fig. 3A). The potential active site is located at the end of the β1 strand (Arg-12 and His-13). In YK23, the active site is covered by two cap domains with flexible loops and short helices: α1 helix with the α1-α2 loop (cap domain 1) and α7, α8, and α9 helices with the connecting loops (cap domain 2) (Figs. 1 and 3). Another extension represents a hairpin-like structure containing β5, α12, β6, and β7 (residues Phe-188 to Leu-231), which wraps around the protein core domain and might be involved in the interaction with other proteins (Figs. 1 and 3). Two YK23 monomers are connected together mainly by hydrophobic interactions between α2, β7, B8, and B9, and the extended C-terminal strand (Val\textsuperscript{47}, Phe\textsuperscript{188}, Leu\textsuperscript{190}, Phe\textsuperscript{230}, Ile\textsuperscript{237}, Leu\textsuperscript{252}, Leu\textsuperscript{254}, and Phe\textsuperscript{258}). This type of dimerization was not observed in other members of the HP family.

A Dali search for YK23 structural homologues identified several homologous structures with the top five matches including the phosphatase Rv3214 from M. tuberculosis (2a6p; Z-score 23.7; r.m.s. deviation 1.9 Å), the phosphoglycerate mutase GpmA from Burkholderia pseudomallei (3gp5; Z-score 20.2; r.m.s. deviation 2.3 Å), the phosphatase PhoE from Bacillus stearothermophilus (1h2e; Z-score 20.1; r.m.s. deviation 2.5 Å), the fructose 2,6-bisphosphatase TIGAR from zebrafish (3e9d; Z-score 20.2; r.m.s. deviation 2.4 Å), the fructose 2,6-bisphosphatase TIGAR from zebrafish (3e9d; Z-score 20.2; r.m.s. deviation 2.4 Å), and the phosphoglycerate mutase GpmA (Rv0489) from M. tuberculosis (1rii; Z-score 19.5; r.m.s. deviation 2.6 Å). Although Rv3214 and PhoE were designated as broad specificity phosphatases (55, 61), Rv3214 showed low activity against the tested natural substrates, whereas PhoE exhibited high activity only on three natural substrates (3-PGA, AMP, and Fru-6P), making their functional annotation as broad specificity phosphatase questionable.

The minimal structure of a histidine phosphatase is represented by the E. coli protein phosphatase SiaX, which has the core domain with the active site covered by the small cap domain comprised of the extended N-terminal strand with a short α-helix (62) (Fig. 3C). All available HP structures show the presence of this small cap domain that probably contributes the residues involved in substrate binding. In YK23, the small cap domain comprise residues Thr\textsuperscript{16}–Thr\textsuperscript{30} (Figs. 1 and 3D). Most structurally characterized HP proteins (e.g. yeast PGMase, rat 2,6-FBPase, rat prostatic acid phosphatase) also have the second, mostly α-helical cap domain inserted into the central part of a protein sequence (residues Tyr\textsuperscript{102}–Asn\textsuperscript{129} in YK23). All HP proteins with the second cap domain are known to function with small substrates (3-phosphoglycerate, Fru-2,6-bisP) suggesting that this domain is likely to be involved in the recognition of small substrates. In addition, the structure of YK23 revealed the presence of a hairpin-like insert domain (Phe\textsuperscript{188}–His\textsuperscript{233}) with two short β-strands (β5 and β6) and α12 helix, which wraps around the core domain and might be involved in the interaction with other proteins (see Figs. 1 and 3D).

Structure of the YK23-FBP Complex—Crystal soaking experiments using crystals of the catalytically inactive YK23 H13A protein and FBP produced a structure of YK23 that revealed the presence of additional electron density bound to the protein active site (PDB code 3L4L, Fig. 4A). This structure was solved to 2.5-Å resolution by molecular replacement using the coordinates of the apo-structure (PDB code 3F3K). This structure superimposes well with the structure of YK23-phosphate complex with r.m.s. deviations between Ca traces of 0.45 Å, a similar distance between two phosphates (10.6 Å), and small shifts...
in the phosphate positions (0.8 Å). The observed additional density was modeled as the linear, extended form of FBP (Fig. 4A) with the cleavable C1-phosphate group positioned in the vicinity of the conserved Arg12 (3.0 Å) and the C6-phosphate between the side chains of Arg181 (2.7 Å) of the same monomer and His244 (3.0 Å) of another monomer (Fig. 4B). Because only a small fraction (2%) of FBP molecules exist in solution in the open form (63), this structure suggests that YK23 binds the cyclic form of the substrate and then converts it into the linear form as was recently proposed for FBP aldolases (64, 65). The cleavable C1-phosphate of FBP is additionally coordinated by the side chains of conserved Arg69 (3.1 Å), Glu99 (3.1 Å), His176 (2.7 Å), and the main chain amide of the signature Gly177 (2.9 Å). In the YK23-FBP complex structure, the last eight residues of the C-terminal tail including His268 were disordered and not modeled. However, comparison with the structure of the YK23 phosphate complex (shown below) suggests that the His268 side chain might interact with the fructose C2 and C4 hydroxyl oxygens (Figs. 4B and 5A). The FBP C2 hydroxyl oxygen is also hydrogen bonded to the side chain of Tyr102 (3.3 Å), whereas the C3 oxygen is near the conserved Arg181 (3.6 Å) and His178 (3.3 Å). Thus, similar to E. coli dPGM or zebrafish TIGAR, the active site of YK23 includes the residues of the signature RHG motif (Arg12, His13, and Gly14), as well as the conserved Arg, Glu (Glu99), and His (His176) suggesting that YK23 employs a similar catalytic mechanism for the hydrolysis of FBP.

**Crystal Structure of YK23 in Complex with Phosphate**—We also solved the structure of YK23 after soaking the apo-protein crystals in a solution of FBP (100 mM) (PDB code 3LG2) by molecular replacement using the coordinates of the apo-structure (PDB code 3F3K). However, this structure revealed the presence of two phosphate mole-

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**FIGURE 3. Crystal structure of YK23.** A, two views of the YK23 monomer related by a 90° rotation. The helices, strands, and loop regions are shown in red, yellow, and green, respectively, and the secondary structure elements are labeled. B, overall structure of the YK23 dimer. Protein monomers are shown in different colors with the labeled secondary structure elements. C and D, comparison of monomer structures of the *E. coli* SixA (C) and YK23 (D). In both structures, the core domain is shown in green, the cap domain-1 in light blue, the cap domain-2 in dark blue, and the hairpin-like insert domain in magenta. In both structures (C and D), the catalytic His is shown as sticks.
cules bound to the protein active site, but no FBP was found (Fig. 5). This can be explained by the presence of significant levels of endogenous orthophosphate in commercial preparations of FBP (0.2 mM phosphate in 100 mM FBP solution). We also found that low millimolar concentrations of phosphate strongly inhibited the hydrolysis of FBP by YK23 (IC\textsubscript{50} \approx 4.5 mM) perhaps by competing with FBP for the active site. Both phosphate molecules are located in the catalytic cleft of YK23 with a distance of 10.1 Å between them and probably mimic two phosphates of the FBP substrate (7.4 Å between the phosphate groups in the cyclic form of FBP and 10 Å in its linear form) (Fig. 5).

The phosphate molecule P1 is located deep in the YK23 active site and probably represents the cleavable C1-phosphate of FBP (Fig. 5, A and B). This phosphate is positioned close to the predicted catalytic His\textsuperscript{13} (2.5 Å), which is a part of the HP signature motif RHG (see Fig. 1). In the \textit{E. coli} phosphoglycerate mutase dPGM, the homologous His residue (His\textsuperscript{15}) has been shown to be transiently phosphorylated during the catalytic cycle (66). In YK23, the P1 phosphate is additionally coordinated by the side chains of conserved Arg\textsuperscript{2} (2.9 Å), Arg\textsuperscript{69} (2.6 Å), Glu\textsuperscript{79} (2.5 Å), and His\textsuperscript{176} (3.0 Å) and the main chain amide group of the conserved Gly\textsuperscript{177} (2.9 Å) (Fig. 5, A and B). In the vicinity of P1, several conserved residues including His\textsuperscript{176}–Ser\textsuperscript{65} (2.7 Å) and His\textsuperscript{178}–Tyr\textsuperscript{102}–Glu\textsuperscript{99} (2.6 Å and 3.5 Å) interact creating a hydrogen bond network that could contribute to substrate coordination and catalysis.

The second phosphate molecule (P2) is located closer to the protein surface at a distance 10.1 Å from P1 and probably indicates the position of the FBP C6-phosphate, which is released with the fructose 6-phosphate product after the reaction. This phosphate is coordinated by the side chains of the conserved His\textsuperscript{178} (2.9 Å) and Arg\textsuperscript{181} (2.4 Å) (Fig. 5A). In addition, this structure revealed that the second monomer contributes two residues coordinating the P2 phosphate bound to the first monomer: the conserved His\textsuperscript{244} (2.7 Å) and semi-conserved His\textsuperscript{268} (2.4–2.7 Å), both located on the extended C-terminal tail (Fig. 5A). In most available structures of HP enzymes (e.g. the phosphoglycerate mutases from \textit{E. coli} and \textit{S. cerevisiae}, or PhoE phosphatase from \textit{B. stearothermophilus}), the C-terminal tails lie along the rim of the active site of the same monomer molecule providing additional stabilizing interactions with the phosphorylated catalytic His or with the bound substrate (42, 54, 66, 67). In fructose 2,6-bisphosphatase from chicken liver, the tail is longer and goes over the entrance twice to the catalytic site essentially blocking substrate access (68). In contrast, the C-terminal tails of the YK23 dimer are located at the dimer interface and come close to the catalytic cleft of another monomer, so that the side chains of two His residues (His\textsuperscript{244} and His\textsuperscript{268}) can make hydrogen bond interactions with the FBP substrate bound to another active site (Fig. 5A). The side chain of His\textsuperscript{268} is positioned below the side chain of the semi-conserved Y24 (3.7 Å) almost between the two phosphate molecules (2.7 Å from P2 and 4.7 Å from P1 oxygens). The His\textsuperscript{244} side chain interacts...
with the carboxylates of the conserved Glu249 (3.3 Å) and semi-conserved Asp270 (3.3 Å) located on the C-terminal tail of the same molecule. Recently, two bound phosphate molecules have also been observed in the structure of another HP enzyme, the fructose 2,6-bisphosphatase TIGAR from zebrafish (52). Although the position of the first phosphate molecule in the TIGAR active site is similar to that of P1 in YK23, the second phosphate is positioned closer (7.5 Å) to the first molecule than in YK23 and is shifted away from the o9-helix compared with YK23 (α11), which can be explained by the different substrate preference of TIGAR (Fru-2,6-bisP). Thus, the YK23 dimer has two symmetrically located composite active sites, which include residues from both monomers.

**Mutational Analysis of YK23 and Potential Catalytic Mechanism**—To identify the residues of YK23 important for the hydrolysis of FBP, we mutated 15 conserved and semi-conserved residues located in the catalytic cavity to Ala and tested the purified mutant proteins for activity against FBP. As expected, loss of the signature residues (Arg12 and His13), as well as the residues forming the YK23 “phosphate pocket” (Glu99 and His176) resulted in mutant proteins with very low or negligible catalytic activity (0.002–0.01 mol/min per mg of protein) (Fig. 6B). In addition, T16A and Y102A mutant proteins also exhibited very low catalytic activity (Fig. 6). The importance of Tyr102 for the activity of YK23 can be explained by its interactions with the FBP C2-hydroxyl and conserved Glu99 and His178, whereas the role of the highly conserved Thr16 is not clear. Thr16 is hydrogen bonded to the side chain of the conserved Ser19 (3.3 Å), and both residues might contribute to the stabilization of the overall shape of the active site cavity (42). Y24A, S65A, and H178A proteins exhibited both low activity and reduced substrate affinity, whereas S19A, R69A, W131A, and R181A retained significant activity with reduced substrate affinity (Table 1). All these residues are located in the YK23 catalytic cavity and interact with FBP (Tyr24, His178, Arg69, and Arg181) or other catalytic residues (Ser65 and Ser19). Interestingly, H178A and R181A also showed reduced activity toward Fru-1P (not shown) suggesting that their role is not limited to the binding of the C6-phosphate of FBP. Whereas the wild-type and all active mutant proteins (Table 1) exhibited a hyper-
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Bolic Michaelis-Menten saturation curve, the R181A protein showed sigmoidal saturation kinetics with the Hill coefficient ($n_H = 1.7$) suggesting positive cooperativity between two subunits in substrate binding. This is similar to the mutation of Lys$^{233}$ to Ala (K233A) in the tyrosyl-tRNA synthetase from B. stearothermophilus, which induced sigmoidal reaction kinetics due to the decreased affinity to ATP (69). Likewise, the R181A protein has a greatly reduced affinity to FBP ($K_m = 1.4\text{ mM}$), which might be sufficient to introduce cooperativity. Alanine replacement-mutagenesis also confirmed the important role of both His$^{244}$ and His$^{268}$, which are located on the C-terminal tail and are involved in the coordination of the FBP C6-phosphate in the active site of another monomer (see Figs. 4B and 5A).

A unique feature of the YK23 FBPase is that it catalyzes a metal-independent hydrolysis of FBP, whereas all known FBPases require a divalent metal cation (usually Mg$^{2+}$) for activity (8–10). The catalytic reaction of the HP family enzymes is based on the transient phosphorylation of a conserved N-terminal His residue preceded by a conserved Arg (involved in phosphate binding) and followed by a conserved Gly, whose carbonyl oxygen coordinates the side chain of the catalytic His (42, 70). These three conserved residues comprise the signature RHG sequence motif of the HP family. In the E. coli phosphoglycerate mutase dPGM, an archetypal HP enzyme, the metal-independent dephosphorylation of substrates (2-phosphoglycerate or 3-phosphoglycerate) involves the transfer of the cleavable phosphate to the conserved His$^{10}$ and the formation of a covalent phosphohistidine intermediate (66). The structure of YK23 and mutational analysis imply that in this protein the cleavable C1-phosphate is coordinated by the side chains of conserved Arg$^{12}$, His$^{13}$, Arg$^{69}$, His$^{176}$, and Glu$^{99}$, which remain bound to the phosphate during its transfer to His$^{13}$ and subsequent hydrolysis of the phosphohistidine intermediate (Fig. 7). The conserved His$^{13}$ is proposed to function as a nucleophile involved in the in-line attack of the C1 phosphoryl group of FBP resulting in the formation of the covalent phospho–His$^{13}$ intermediate (Fig. 7). The side chain of Glu$^{99}$ is located deep in the active site and its environment is quite hydrophobic suggesting that it would remain protonated in the YK23-FBP complex. In the first reaction step (FBP hydrolysis and formation of a phospho–His intermediate), Glu$^{99}$ can serve as a proton donor for the leaving Fru-6P (the first reaction product), whereas the cleaved C1-phosphate is transferred to His$^{13}$ (Fig. 7). During the second reaction step, hydrolysis of the phospho–His$^{13}$, the unprotonated side chain of Glu$^{99}$ activates a bound water molecule for a nucleophilic attack on the phospho–His$^{13}$ intermediate producing free phosphate as the second reaction product (Fig. 7). In the YK23 apo-structure (3F3K), a water molecule is suitably positioned between the Glu$^{99}$ (2.6 Å) and His$^{13}$ (4.6 Å). In phosphatases producing a phosphohistidine intermediate during the reaction, the hydrolysis of this intermediate often represents a rate-limiting step (71). If this is also true for YK23, then this enzyme would be expected to exhibit biphasic kinetics of FBP hydrolysis.

CONCLUSIONS

Thus, the HP family protein YK23 efficiently hydrolyzes FBP in vitro using a metal-independent catalytic mechanism, whereas all known FBPases from the lithium-sensitive phosphatase superfamily employ a different, metal-dependent mechanism. This is similar to the hydrolysis of a phosphodiester bond by different families of restriction endonucleases and phospholipases D, which can use metal-dependent or metal-independent reaction mechanisms (72, 73). Interestingly, in all these enzymes the metal-independent phosphoesterase mechanism is based on the formation of a covalent phosphohistidine intermediate. The presence of a metal-independent enzymatic pathway in a living organism might be beneficial under conditions of metal limitation or inhibition.

S. cerevisiae is known to have one conventional FBPase (FBP1, type-I), which is induced during growth on gluconeogenic substrates and rapidly degraded upon glucose addition (74). Because YK23 has lower substrate affinity to FBP than FBP1, it is not likely to be able to substitute FBP1 in gluconeogenesis under normal growth conditions. Indeed, the S. cerevisiae fbp1 mutant strain has been shown to be a hexose auxotroph and did not grow on gluconeogenic substrates in minimal medium (75). However, in S. cerevisiae YK23 might replace FBP1 in the gluconeogenic pathway under conditions of metal limitation. In other organisms, the YK23 orthologues might function as alternative FBPases, because they exhibit the substrate affinity and catalytic efficiency similar to that of metal-dependent FBPases. Recently, it has been shown that the FBP1 deletion strain of the non-conventional yeast Yarrowia lipolytica can grow on gluconeogenic substrates (like ethanol) suggesting the presence of another FBPase (76). The Y. lipolytica genome encodes a YK23 orthologue, YALI0D9229 (Q6C9Q2, 42% sequence identity to YK23), which has all residues important for the FBPase activity of YK23 conserved (Arg$^2$, His$^{10}$, Thr$^{13}$, Ser$^{62}$, Glu$^{95}$, Tyr$^{96}$, His$^{174}$, His$^{176}$, and His$^{210}$) (Fig. 1), suggesting that this protein might be an alternative FBPase. Thus, the crystal structure of YK23 defined structural determinants for substrate recognition and the catalytic mechanism of a novel, metal-independent FBPase family. It seems that eukaryotic cells, like prokaryotes, have multiple FBPases, and future studies are required to identify their physiological function.

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