Structure-based Functional Annotation

YEAST ymr099c CODES FOR A d-HEXOSE-6-PHOSPHATE MUTAROTASE*

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Despite the generation of a large amount of sequence information over the last decade, more than 40% of well characterized enzymatic functions still lack associated protein sequences. Assigning protein sequences to documented biochemical functions is an interesting challenge. We illustrate here that structural genomics may be a reasonable approach in addressing these questions. We present the crystal structure of the Saccharomyces cerevisiae YMR099cp, a protein of unknown function. YMR099cp adopts the same fold as galactose mutarotase and shares the same catalytic machinery necessary for the interconversion of the α and β anomers of galactose. The structure revealed the presence in the active site of a sulfate ion attached by an arginine clamp made by the side chain from two strictly conserved arginine residues. This sulfate is ideally positioned to mimic the phosphate group of hexose 6-phosphate. We have subsequently successfully demonstrated that YMR099cp is a hexose-6-phosphate mutarotase with broad substrate specificity. We solved high resolution structures of some substrate enzyme complexes, further confirming our functional hypothesis. The metabolic role of a hexose-6-phosphate mutarotase is discussed. This work illustrates that structural information has been crucial to assign YMR099cp to the orphan EC activity: hexose-phosphate mutarotase.

The atomic coordinates and structure factors (code 2CIQ, 2CIR, and 2CIS) were deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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YMR099cp was expressed in Escherichia coli strain DE3 with 50 μg/ml kanamycin at 37 °C using the transformed cloned into a derivative of pET9 vector (Stratagene). Expression was done at 37 °C using the transformed Escherichia coli Gold (DE3) strain and 2xYT medium (BIO101 Inc.) supplemented with kanamycin at 50 μg/ml. Cells were harvested by centrifugation, resuspended in 30 ml of 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol, and stored overnight at −20 °C. Cell lysis was completed by sonication. The His-tagged protein was purified on a Ni-nitrilotriacetic acid column (Qiagen Inc.) followed by gel filtration on a SuperdexT M200 column (Amersham Biosciences).

**Glucose-6-phosphate Epimerase Activity Measurements**

Because of the specificity of glucose-6-phosphate dehydrogenase for β-Glc6P, both spontaneous and YMR099cp-catalyzed formation of β-Glc6P could be followed by measuring the increase in absorbance at 340 nm caused by generation of NAD(P)H, coupled to the conversion of glucose-6-phosphate dehydrogenase. All kinetic experiments were performed at 25 °C in 50 mM imidazole, pH 7.6, 50 mM KCl, 8 mM MgSO4 using an Applied Photophysics SW18-MV stopped-flow spectrophotometer. Reactions were initiated by mixing equal volumes of cono-1,5-lactone 6-phosphate by glucose-6-phosphate dehydrogenase from S. cerevisiae and 2 mM NADP+. 1D-1H NMR was acquired with 16,000 data points and 16 scans. Two-dimensional NOESY was recorded to a crystallization solution with progressively higher glycerol concentrations up to 30% v/v (unbound form) and then flash-cooled in liquid nitrogen. Diffraction data were collected from a flash-cooled crystal at 100 K on beamline ID23-1 at the European Synchrotron Radi-

| Data collection | Apo form | Glc6P form | Gal6P form |
|----------------|---------|-----------|----------|
| Resolution (Å) | 20.1-7  | 20.1-6    | 20.1-6   |
| Space group    | P2₁̅₂̅₂ | C222      | C222     |
| Unit cell parameters (Å) | a = 44.9; b = 74.2; c = 106.5 | a = 43.7; b = 157.3; c = 104.2 | a = 43; b = 156.3; c = 104.0 |
| Total number of reflections | 137,453 | 248,220   | 204,046  |
| Total number of unique reflections | 39,082 | 57,846    | 43,501   |
| Completeness (%) | 6.1 (40.7) | 7.8 (46.3) | 6.3 (20.7) |
| I/s(I) | 14.8 (98.2) | 98.9 (99.6) | 93 (71.8) |
| Redundancy | 3.5 | 4.3 | 4.7 |
| Ramachandran plot (%) | 88.9 | 89 | 88.6 |
| Most favored | 11.1 | 11 | 11.4 |
| Generously allowed | 2CIQ | 2CIR | 2CIS |

The abbreviations used are: Gal6P, d-galactose 6-phosphate; Glc6P, d-glucose 6-phosphate; RMSD, root-mean-square deviation; NOESY, nuclear Overhauser effect spectroscopy.
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The crystal structure of the S. cerevisiae gene product YMR099cp (297 amino acids) has been solved using the molecular replacement method using the structure of a protein of unknown function (H. influenzae Hi1317; PDB code: 1JOV) and refined to 1.7-Å resolution (Table 1). All 2Fo–Fc electron density maps are of excellent quality, except for the N-terminal methionine and 9 C-terminal amino acids that have not been built because they were not defined in electron density. One copy of YMR099cp is present in the asymmetric unit, confirming gel filtration elution profile that clearly indicates a monomeric state of the protein in solution (data not shown). YMR099cp is made of a single globular domain of approximate dimensions 37 × 52 × 53 Å³. Its structure can be described as a β-sandwich made of 22 β-strands and two short α-helices (Fig. 1A). Strands are organized in four anti-parallel β-sheets arranged in two parallel layers. The first one is made of three sheets: S1 composed of β-strands 1–4, S2 (strand order: β5, β10, and β21), and S4 (strand order: β11, β20, β19, β16, β13, and β14). The second layer is formed by the sole 9-stranded β-sheet S3 (strand order: β6–β9, β22, β18, β17, β12, and β15). The two helices α1 and α2 are facing toward the same side of the monomer and are part of the linkers connecting strands β7 to β8 and β19 to β20. This fold has already been described for galactose mutarotases (RMSD of 2.2–2.6 Å over 240 Ca positions; 15–17% sequence identity (11, 20, 21)), domain 5 of β-galactosidase (RMSD of 3.1 Å over 190 Ca positions, 7% sequence identity (22)) and the N-terminal domain of maltose phosphorylase (RMSD of 3.4 Å over 164 Ca positions, 13% sequence identity (23)). Structural similarity is also shared with proteins of unknown function: Caenorhabditis elegans C01B4.6 gene product (RMSD of 2.41 Å over 235 Ca positions, the asymmetric unit, confirming gel filtration elution profile that clearly indicates a monomeric state of the protein in solution (data not shown). YMR099cp is made of a single globular domain of approximate dimensions 37 × 52 × 53 Å³. Its structure can be described as a β-sandwich made of 22 β-strands and two short α-helices (Fig. 1A). Strands are organized in four anti-parallel β-sheets arranged in two parallel layers. The first one is made of three sheets: S1 composed of β-strands 1–4, S2 (strand order: β5, β10, and β21), and S4 (strand order: β11, β20, β19, β16, β13, and β14). The second layer is formed by the sole 9-stranded β-sheet S3 (strand order: β6–β9, β22, β18, β17, β12, and β15). The two helices α1 and α2 are facing toward the same side of the monomer and are part of the linkers connecting strands β7 to β8 and β19 to β20. This fold has already been described for galactose mutarotases (RMSD of 2.2–2.6 Å over 240 Ca positions; 15–17% sequence identity (11, 20, 21)), domain 5 of β-galactosidase (RMSD of 3.1 Å over 190 Ca positions, 7% sequence identity (22)) and the N-terminal domain of maltose phosphorylase (RMSD of 3.4 Å over 164 Ca positions, 13% sequence identity (23)). Structural similarity is also shared with proteins of unknown function: Caenorhabditis elegans C01B4.6 gene product (RMSD of 2.41 Å over 235 Ca positions,
13% sequence identity; PDB code: 1LUR) and *S. cerevisiae* YMR071cp (RMSD of 2.67 Å over 243 Cα positions, 12% sequence identity; PDB code: 1YGA).

**Comparison with Galactose Mutarotases**—The crystal structures of YMR099cp and of the *H. influenzae* HiI1317 protein (which provided the model used for molecular replacement) confirm the Psi-Blast prediction that they belong to the aldose-1-epimerase family. The best structurally and biochemically characterized member of this family is galactose mutarotase, an enzyme that catalyzes the conversion of \(\alpha\)-D-galactose (24). This is the first step of the Leloir pathway, which in most organisms converts \(\beta\)-galactose to the more metabolically useful glucose 1-phosphate.

Surface projection of sequence conservation among YMR099cp-related proteins has highlighted a highly conserved pocket whose floor is made by sheet S4 and walls by helix α2 as well as by the long loop connecting β4 to β6 (Fig. 1B). This pocket, which contains a glycerol-bound molecule bound in the so-called apo structure, corresponds to the active site in galactose mutarotase. Sequence alignment of YMR099cp orthologs and galactose mutarotases reveals five strictly conserved pocket residues (Figs. 1C and 2): two histidine residues (His82 and His159, YMR099cp numbering), two acidic amino acids (Asp203 and Glu264) and Tyr161. Crystal structures of *Lactococcus lactis* galactose mutarotase bound to sugars have shown that these residues are involved in sugar binding (11, 25). Site-directed mutagenesis led to the conclusion that in *L. lactis* galactose mutarotase, His170 and Glu304 play the role of catalytic acid and base, respectively (26). Hence, YMR099cp seems to possess the catalytic machinery necessary to interconvert \(\alpha\) and \(\beta\) sugar anomers, suggesting that YMR099cp is a yeast galactose mutarotase.

However, Gal10p was annotated recently with this activity in *S. cerevisiae* (27). Gal10p is a bifunctional enzyme with the N-terminal domain harboring UDP-galactose 4-epimerase and the C-terminal domain galactose mutarotase activities (21). This raises the issue whether multiple genes code for galactose mutarotase activity in yeast or YMR099cp is an active mutarotase with different substrate specificity. An indication supporting the latter hypothesis comes from the refinement of the native structure, which has revealed a sulfate ion bound in the active site pocket (Fig. 1C). This ion, present in the crystallization buffer, is complexed by two arginine side chains (Arg57 and Arg86) that are strictly conserved among YMR099cp orthologs but not among bona fide galactose mutarotases (Fig. 2, family 1, black arrows). Interestingly, a sulfate ion occupies exactly the same position in

![FIGURE 2. Structure-based sequence alignment of YMR099cp/galactose mutarotase enzymes. *S. cerevisiae* YMR099cp and *L. lactis* galactose mutarotase secondary structure elements are shown above and below the sequences, respectively. Strictly conserved residues are in white on a black background. Partially conserved amino acids are boxed. The two conserved Arg of the arginine clamp involved in sulfate/phosphate binding are indicated by arrows.](image-url)

![FIGURE 3. Glucose-6-phosphate mutarotase activity assay. A, schematic representation of the coupled enzyme assay used to detect the glucose-6-phosphate mutarotase activity. B, stopped-flow experiments. All measurements were performed at 25 °C in 50 mM imidazole, pH 7.6, 50 mM KCl, 8 mM MgSO₄. Reactions were initiated by mixing equal volumes of 60 μM equilibrated Glc6P and 2 mM NADP⁺ with 160 units/ml glucose-6-phosphate dehydrogenase from *L. mesenteroides*, 2 mM NAD⁺, and various concentrations of YMR099cp. Inset, first order velocity constants \(k_{1}\), of the conversion of \(\alpha\)-Glc6P to \(\beta\)-Glc6P is plotted as a function of protein concentration. Values obtained in this study (triangles) and by Wurster and Hess (squares; Ref. 12) are compared.](image-url)
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the active site of *H. influenzae* H11317 (unknown function, PDB code: 1JOV), and is liganded by homologous arginines. Finally, structure superimposition of the glucose-bound galactose mutarotase E304Q mutant of *L. lactis* onto YMR099cp shows that the glucose O₆ atom and an oxygen atom from sulfate almost overlap. The fact that sulfate ions often substitute for phosphate groups in protein structures (28, 29) suggests that YMR099cp binds a hexose-6-phosphate and that it hence may have hexose-6-phosphate mutarotase (or 1-epimerase) activity. A glucose-6-phosphate-1-epimerase, which catalyzes the mutarotation of d-glucopyran 6-phosphate (12−15) was previously purified from yeast, but the sequence of the protein was not identified. Interestingly, the previously published data for the enzyme purified from yeast cells (estimated molecular mass of 35-kDa and isoelectric point of 5.8; Ref. 12) fit very well with the values calculated from the YMR099cp sequence (34 kDa and pI of 6.07).

Functional Studies—These structural observations led us to test YMR099cp for hexose 6-phosphate epimerase activity. For this purpose, we have used an enzyme-coupled assay. It consists in following the reduction of NAD(P) (at 340 nm) resulting from glucose-6-phosphate dehydrogenase activity, which specifically converts β-Glc6P (but not the α form) into d-glucono-1,5-lactone 6-phosphate (Fig. 3A). The formation of β-Glc6P from a racemic mixture has been investigated by stopped-flow at various YMR099cp concentrations (Fig. 3B), which revealed two distinct phases. The first one corresponds to the initial burst of β-Glc6P because of its presence in the racemic mixture and hence is independent on the YMR099cp concentration. In the second phase, faster generation of NAD(P)H reflecting higher velocity constant (kₐ) for the interconversion between glucose 6-phosphate anomers is obtained with increasing concentration of YMR099cp, demonstrating the glucose-6-phosphate epimerase activity of the protein (Fig. 3B, inset). In our experiments, YMR099cp accelerates the reaction two to three times more than previously observed in the same conditions by Wurster and Hess (Fig. 3B, inset; Ref. 12). This probably reflects the general improvement in the quality (i.e. purity and stability) of a protein sample extracted and purified within a few hours from an overexpressing *E. coli* strain, compared with the one of a protein sample obtained from a wild-type yeast lysate using a long and tedious purification protocol (12).

Unfortunately, this enzymatic assay could not be used to test other hexose 6-phosphate substrates because glucose-6-phosphate dehydrogenase exhibits a high degree of specificity for β-Glc6P. A more common way to measure epimerase activity is polarimetry, but this method requires the pure α or β anomeric forms of the substrates, which are usually not commercially available. We therefore used NMR to identify activity on other hexose 6-phosphate substrates (30). To identify putative substrates to be tested by NMR, we have first tested the binding of a few potential ligands using tryptophan fluorescence. As the comparison of the YMR099cp and galactose mutarotase active sites revealed that the aromatic side chain (phenylalanine or tyrosine) that forms a hydrophobic sugar binding platform in galactose mutarotases is replaced by a strictly conserved tryptophan residue in all YMR099cp homologs, we then assumed that this side chain could be used as a fluorescent probe. This allowed us to test the binding affinity of several sugar candidates as a ligand for YMR099cp. In addition to glucose 6-phosphate, shown to be a substrate of the protein, we tested the binding of glucose, three additional hexose 6-phosphate sugars (Gal6P, Man6P, and Fru6P) and ribose 5-phosphate. These measurements revealed that YMR099cp displays a slightly higher affinity for Fru6P and Man6P than for Glc6P (Kᵢ of 114 μM, 160 μM, and 200 μM, respectively) and has lower affinity for Rib5P and Gal6P (1−mm, Table 2). Interestingly, the Kᵢ value of 200 μM measured for Glc6P by fluorescence is comparable to the Kᵢ value (144 μM) determined for yeast glucose-6-phosphate epimerase (15). No binding could be detected for glucose demonstrating the importance in substrate specificity of the phosphate group.

We have tested both Man6P and Gal6P as mutarotase substrates of YMR099cp using NMR techniques. Man6P and Gal6P differ from Glc6P by the orientation of the hydroxyl group at position C₂ and C₄, respectively (Fig. 4). First, sugar binding has been monitored using a ¹H-¹D reference spectrum and a one-dimensional saturation difference (STD) technique (31). The ¹H-¹D reference spectra recorded for these different ligands (present at 20 mM) in the absence or presence of 25 μM YMR099cp show a specific line broadening for the peaks corresponding to both the α and β anomeric protons upon enzyme addition. This indicates that YMR099cp binds to both anomeric forms from Glc6P, Gal6P, and Man6P (Fig. 5). This was confirmed by the STD spectra recorded for these three sugars (data not shown). Second, enzyme catalyzed anomeric interconversion was investigated using two-

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**TABLE 2**

**Ligand binding**

| Ligand             | Kᵢ (μM) |
|--------------------|---------|
| Glucose 6-phosphate| 0.2     |
| Mannose 6-phosphate| 0.16    |
| Galactose 6-phosphate| 0.985  |
| Ribose 5-phosphate | 1.1     |
| Fructose 6-phosphate| 0.114   |
| Glucose            | ND*     |

**Notes:**

* ND, not detectable.

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**FIGURE 4.** Schematic representations of phosphosugars used in this study.
FIGURE 5. Hexose-6-phosphate mutarotase activity of YMR099cp. Two-dimensional NOESY spectra recorded with 200-ms mixing time were obtained for Glc6P (A), Gal6P (B), and Man6P (C) in the absence (left column) or presence (right column) of YMR099cp. The 1H one-dimensional NMR spectrum for each sample is shown on the x- and y-axis of the NOESY spectra. Exchange cross-peaks induced by addition of YMR099cp are linked to peaks from α and β anomic protons by dashed lines.
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A.

FIGURE 6. Complexes of YMR099cp bound to hexose 6-phosphate sugars. Stereoview representation of Glc6P (A) and Tag6P (B) bound into the YMR099cp active site. The 2F o – F c electron density maps contoured at 1σ are shown in blue around the ligands. Hydrogen bonds made by the ligands with YMR099cp as well as Ba2+ Tag6P oxygen coordination are depicted by red dashed lines. Water molecules are shown by red spheres.

dimensional nuclear Overhauser effect spectroscopy (NOESY) as previously described for E. coli RbsD, a protein with mutarotase and pyranase activities on d-ribose (32). Exchange cross-peaks between α and β Glc6P anomers could only be observed in the presence of YMR099cp enzyme (Fig. 5A). These cross-peaks are due to a fast YMR099cp-catalyzed interconversion between α and β Glc6P, thus confirming the glucose-6-phosphate mutarotase activity measured by enzyme-coupled assay. In addition, this validates that NOESY experiments are useful to screen for putative YMR099cp substrates. NOESY exchange cross-peaks between anomers were also observed on Gal6P and Man6P, when mixed with YMR099cp (Fig. 5, B and C). This clearly shows that YMR099cp also has mutarotase activity on Gal6P and Man6P sugars.

Altogether, these functional data clearly demonstrate that YMR099cp catalyzes the interconversion between the α and β anomers from at least three hexose 6-phosphate sugars (Glc6P, Gal6P, and Man6P). In addition, the velocity constant measured for the YMR099cp-catalyzed anomerization of α to β Glc6P (k−1 = 62.8 min−1 with 1.72 μM YMR099cp) and the affinity constant determined for Glc6P (Kd = 200 μM) are comparable to those of the glucose-6-phosphate epimerase initially described by Wurster and Hess (12–14) in identical experimental conditions (k−1 = 32 min−1 with 1.72 μM enzyme and Kd = 55 μM and 144 μM for β and α anomers, respectively).

Complexes with Hexose 6-Phosphate Sugars—To get a better understanding of the substrate preference of YMR099cp, we have solved the structures of its complexes with Glc6P and Gal6P to 1.6-Å resolution (Fig. 6). Virtually no conformational changes are observed between the free and complexed YMR099cp (RMSD value of 0.14–0.22 Å between the various structures).

For Glc6P, the 2F o – F c electron density map demonstrated that only the β anomer of the pyran form is bound and that the sugar ring adopts a chair conformation. The sugar phosphate moiety makes one hydrogen bond with the Gln81 side chain Ne2 atom and is bound by the positively charged side chains from Arg57 and Arg86 as observed for the sulfate ion in the apoprotein (Fig. 6A). These two residues form an arginine clamp only conserved in YMR099cp orthologs. This arginine clamp could be the hallmark distinguishing mutarotases acting on hexose 6-phosphate from those acting on hexoses (no binding to glucose could be detected by fluorescence measurements). The sugar ring is sandwiched between Gln81 and Trp238 side chains. The O2, O4 hydroxyl groups, and O1 ring sugar from Glc6P are hydrogen-bonded to Asp203 Oδ2, Lys244 Nε2, and His82 Ne2, respectively. In addition, water-mediated hydrogen bonds connect the phosphate group to Tyr161 Oη and Glc6P O5 to Asp203 Oδ1. Finally, minor contacts are observed between Glc6P and Phe67, Gln183, and Met248.

In the unbound structure of YMR099cp, the strictly conserved His159 and Glu264 perfectly superpose with the catalytic residues identified in L. lactis galactose mutarotase (His170 and Glu304, see above). Structural and enzymatic studies on L. lactis enzyme mutants bound to galactose have unambiguously shown that His170 acts as the active site acid by protonating the O5 sugar ring oxygen (distance between His170 Ne2 and O5 atoms is 3 Å) while Glu304 is ideally located (its Oε1 and Oε2 oxygens are 2.7 Å away from galactose O5 atom) to act as general base through deprotonation of the anomic O1 hydroxyl group (26). This led us to assume that the anomerization of hexose 6-phosphate by YMR099cp proceeds via the same catalytic mechanism as galactose mutarotase and that His159 and Glu264 are the catalytic acid and base, respectively. In our YMR099cp-Glc6P complex, these two residues adopt the same conformations as in the galactose mutarotase-galactose complex. However, they make no hydrogen bonds with either the O1 or O3 Glc6P oxygen atoms, because it is less deeply buried into the active site pocket (Fig. 5A). Glc6P therefore probably forms a non-productive complex in our YMR099cp crystal. A similar observation was made for crystal structure of the wild-type and E304Q mutant forms of L. lactis galactose mutarotase, which both bind only the β-anomer of glucose in a non-productive manner (25, 26). On the contrary, the natural substrate galactose is well positioned in the active site and a mixture of
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both anomers is observed ($k_{cat}/K_m = 185$ and $12.65 \text{ s}^{-1}\text{ mM}^{-1}$ for galactose and glucose, respectively (26)). Because there exists no biochemical assay to measure the mutarotation of Man6P or Gal6P (and their pure anomers are commercially unavailable) we were not able to determine their kinetic parameters and we therefore cannot be sure of the preferred substrate of YMR099cp (the affinity for Man6P ($K_{d}$ 0.16 mm) is higher than for Glc6P ($K_{d}$ 0.2 mm).

Surprisingly, the 1.6-Å resolution structure of the YMR099cp Gal6P complex unambiguously revealed that it is the β form of tagatose 6-phosphate (Tag6P), the Gal6P isomer (Fig. 6B), which is present in the active site. As observed for Glc6P, the 'Tag6P phosphate moiety interacts with the Glu81, Arg57, and Arg86 side chains from the arginine clamp and the furan ring packs on Trp238. The Tag6P O3, O4, and O5 atoms are H-bonded to Asp203 O, and Glu304 and His170 act as catalytic base and acid, respectively (26). Glu304 starts the reaction by protonating the C-5 ring oxygen. A growing number of mutarotases acting on a wide variety of furan or pyran sugars ((deoxy)ribose, fucose, rhamnose) has been characterized recently (30, 39, 40). Here, we bring structure-based experimental evidence that the YMR099cp exhibits hexose-6-phosphate mutarotase activity on Glc6P, Gal6P, and Man6P. Glc6P mutarotase activity was previously described for an unidentified enzyme purified from wild-type yeast exhibiting a similar molecular weight and pI as YMR099cp (12–14). It was shown that specific anomers of both Man6P and Glc6P are subject to metabolic conversions. For instance, yeast phosphomannose isomerase, which catalyzes the interconversion of Man6P and Fru6P, is specific for the Man6P α-anomer but both mannose anomers are phosphorylated by hexokinase (41). Hence, the mutarotase activity that we have observed on Man6P could provide sufficient amounts of β-Man6P for phosphomannose isomerase. The need for an enzyme interconverting α- and β-Glc6P anomers is further justified by the specificity of different enzymes involved in sugar metabolism pathways (Fig. 7). First, glucose-6-phosphate dehydrogenase specifically occurs spontaneously in solution, the in vivo rate may be too low to support fast energy generation by metabolic pathways (the rates for spontaneous mutarotation of glucose and glucose-6-phosphate are 0.015 min$^{-1}$ and 0.09 min$^{-1}$, respectively (35)). Hence, aldose-1-epimerases (or mutarotases; EC 5.1.3.3) increasing the rate of the anomerization are required. The most studied enzyme of this family, galactose mutarotase catalyzes the first of the four steps of the Leloir pathway which converts β-D-galactose (one of the lactose degradation products with α-D-glucose) into the metabolically more useful glucose 1-phosphate (24, 36). This first step consists of the interconversion of β-D-galactose to α-D-galactose, which is then phosphorylated by galaktokinase to yield α-D-galactose 1-phosphate. The latter is transformed into UDP-glucose by galactose-1-phosphate uridylyltransferase to produce glucose 1-phosphate and UDP-galactose. Finally, UDP-galactose 4-epimerase regenerates UDP-glucose from UDP-galactose. In human, mutations in the genes encoding for either of the four enzymes involved in the Leloir pathway result in galactosemia, a rare but potentially lethal disease leading to cataract formation and liver dysfunction (37, 38).

In recent years, the galactose mutarotase catalytic mechanism has been dissected by the resolution of the crystal structures of the L. lactis enzyme bound to different substrates combined with site-directed mutagenesis studies (11, 25, 26). A two-layered β-sandwich fold made of 20–30 antiparallel β-strands was revealed for L. lactis, human and yeast enzymes (11, 20, 21). In addition, the structure of the galactose-bound enzyme has highlighted four strictly conserved residues (2 histidines and two acidic amino acids) located in the galactose binding pocket that could potentially act as general acid/base in the mutarotation reaction. Substitution of the acidic residues (Asp243 and Glu304 according to L. lactis numbering) by Ala and of two histidines (His96 and His170) by Asn has lead to the conclusion that Glu304 and His170 act as catalytic base and acid, respectively (26). Glu304 starts the reaction by abstracting the proton from the C-1 hydroxyl group of the sugar while His170 protonates the C-5 ring oxygen. A growing number of mutarotases acting on a wide variety of furan or pyran sugars ((deoxy)ribose, fucose, rhamnose) has been characterized recently (30, 39, 40). Here, we bring structure-based experimental evidence that the YMR099cp exhibits hexose-6-phosphate mutarotase activity on Glc6P, Gal6P, and Man6P. Glc6P mutarotase activity was previously described for an unidentified enzyme purified from wild-type yeast exhibiting a similar molecular weight and pI as YMR099cp (12–14). It was shown that specific anomers of both Man6P and Glc6P are subject to metabolic conversions. For instance, yeast phosphomannose isomerase, which catalyzes the interconversion of Man6P and Fru6P, is specific for the Man6P α-anomer but both mannose anomers are phosphorylated by hexokinase (41). Hence, the mutarotase activity that we have observed on Man6P could provide sufficient amounts of β-Man6P for phosphomannose isomerase. The need for an enzyme interconverting α- and β-Glc6P anomers is further justified by the specificity of different enzymes involved in sugar metabolism pathways (Fig. 7). First, glucose-6-phosphate dehydrogenase specifically
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catalyzes the conversion of the Glc6P β-anomer into d-gluc-
cono-1,5-lactone-6-phosphate. This is the first irreversible and
rate-limiting step of the pentose phosphate pathway that gen-
erates NAD(P)H from NAD(P)⁺ upon conversion of β-Glc6P
into Fru6P (42). Second, the Glc6P α-anomer is the specific
substrate for both phosphoglucomutase and phosphoglucose
isomerase. Phosphoglucomutase is involved in glycolysis as
well as in the metabolism of glycogen, trehalose, and galactose
and also catalyzes the interconversion of α-d-Glc1P and α-d-
Glc6P (43). Phosphoglucose isomerase converts α-d-Glc6P to
Fru6P during glycolysis and the reverse reaction during glu-
coneogenesis (43). Enzyme with specificity for Gal6P anomers
has not been described yet and whether Gal6P is a biological
substrate of YMR099cp remains an open question. Surpris-
ingly, the complex between YMR099cp and Gal6P revealed the
presence of the furanose isomer of Gal6P (Tag6P) in the crys-
tals. The exclusive presence of Tag6P in the YMR099cp active
site can be explained by the higher affinity of the protein for the
furanose form of the substrate, at least in the crystal. It should
be noted that YMR099cp exhibits higher affinity for the fura-
nose Fru6P ($K_d$ (p) $114 \mu M$) than for the pyran form of Gal6P ($K_d$ (p) $985 \mu M$). The Tag6P bound to YMR099cp active site is very
likely to mimic Fru6P. Analysis of our complexes (Fig. 6) clearly
shows that the main interaction with the substrates occurs
through anchoring of the 6 phosphate group and that the active
site pocket can easily accommodate various isomers at posi-
tions 2 and 4 on the sugar ring. This explains why we observe
such broad substrate specificity. Whether YMR099cp acts in a
precise metabolic pathway, like galactose mutarotase does,
remains an open question.

In conclusion, it has been recently reported that 40% of the
enzymatic activities described by EC numbers are not associ-
ated with any protein sequence in major public databases. In
this article we demonstrate that a systematic structural
approach can help to fill this gap in favorable cases. We show
here that the S. cerevisiae ymr099c gene product codes for an
enzyme that corresponds to a hitherto orphan EC number (EC
5 1.3.15). The definition of the physiological role of the enzyme
will need further exploration. Hopefully, this will explain why
YMR099c belongs to the 1% of the genes regulated by the tran-
scription activator GCN4 that, to a vast majority, encode for
proteins involved in amino acid and nucleotide metabolism (7).
Similarly, it remains to be determined if the co-purification of
YMR099cp with TRZ1, a tRNA 3′ processing endonuclease
responsible for a 3′ trailer from precursor tRNA (8–10), as any
biological relevance or if this is an artifact.

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REFERENCES
1. Karp, P. D. (2004) Genome Biol. 5, 401
2. Lespinet, O., and Labedan, B. (2005) Science 307, 42
3. Roberts, R. J. (2004) PLoS Biol. 2, E42
4. Kuhn, P., Lesley, S. A., Mathews, I. M., McMullan, D., McPhilips, T. M., Miller, M. A., Miller, M., Morse, A.,
Moyle, K., Ouyang, J., Robb, A., Rodriguez, K., Selby, T. L., Spraggon, G., Stevens, R. C., Taylor, S. S., van den Bedem, H., Velasquez, J., Vincent, J. W., West, B., Wolf, G., Wooley, J., and Wilson, I. A. (2002) Proteins 49, 142–145
5. Mathews, I., Deacon, A. M., Canaves, I. M., McMullan, D., Lesley, S. A., Agarwalla, S., and Kuhn, P. (2003) Structure 11, 677–690
6. Quevillon-Cheruel, S., Liger, D., Leulliot, N., Galle, M., Poupon, A., de La Sierra-Gallay, I. L., Zhou, C. Z., Collinet, B., Janin, J., and Van Tilburg, H. (2004) Biochimie. (Paris) 86, 617–623
7. Schuldiner, O., Yanover, C., and Benvenisty, N. (1998)Curr. Genet. 33, 16–20
8. Hazbun, T. R., Malmstrom, L., Anderson, S., Graczyk, B. J., Fox, B., Riffle, M., Sundin, B. A., Aranda, J. D., McDonald, W. H., Chiu, C. H., Snydman, B. E., Bradley, P., Muller, E. G., Fields, S., Baker, D., Yates, J. R., 3rd, and Davis, T. N. (2003) Mol. Cell 12, 1353–1365
9. Takaku, H., Minagawa, A., Takagi, M., and Nashimoto, M. (2003) Nucleic Acids Res. 31, 2277–2278
10. Chen, Y., Beck, A., Davenport, C., Shattuck, D., and Tavtigian, S. V. (2005) BMC Mol. Biol. 6, 12
11. Thoden, J. B., and Holden, H. M. (2002) J. Biol. Chem. 277, 20854–20861
12. Wurster, B., and Hess, B. (1972) FEBS Lett. 23, 341–344
13. Wurster, B., and Hess, B. (1974) Hoppe Seylers Z. Physiol. Chem. 355, 255–260
14. Wurster, B., and Hess, B. (1975) Methods Enzymol. 41, 488–493
15. Chance, E. M., Hess, B., Plessner, T., and Wurster, B. (1975) Eur. J. Biochem. 50, 419–424
16. Kabosch, W. (1993) J. Appl. Crystallogr. 26, 795–800
17. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
18. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458–463
19. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997)Acta Crystallogr. D Biol. Crystallogr. 53, 240–255
20. Thoden, J. B., Timson, D. J., Reece, R. J., and Holden, H. M. (2004) J. Biol. Chem. 279, 23431–23437
21. Thoden, J. B., and Holden, H. M. (2005) J. Biol. Chem. 280, 21900–21907
22. Jacobson, R. H., Zhang, X. J., Dubose, R. F., and Matthews, B. W. (1994) Nature 369, 761–766
23. Egloff, M. P., Uppenberg, J., Haalck, L., and van Tilburgh, H. (2001) Structure (Camb) 9, 689–697
24. Holden, H. M., Raymond, I., and Thoden, J. B. (2003) J. Biol. Chem. 278, 43885–43888
25. Thoden, J. B., Kim, J., Rauhfeld, F. M., and Holden, H. M. (2002) J. Biol. Chem. 277, 45458–45465
26. Thoden, J. B., Kim, J., Rauhfeld, F. M., and Holden, H. M. (2003) Protein Sci. 12, 1051–1059
27. Majumdar, S., Ghatak, J., Mukherji, S., Bhattacharjee, H., and Bhaduri, A. (2004) Eur. J. Biochem. 271, 753–759
28. Li de la Sierra, I., Munier-Lehmann, H., Gilles, A. M., Barzu, O., and Delarue, M. (2001) J. Mol. Biol. 311, 87–100
29. Li de la Sierra-Gallay, I., Collinet, B., Galle, M., Quevillon-Cheruel, S., Liger, D., Minard, P., Blondeau, K., Heneces, G., Aufrere, R., Leulliot, N., Zhou, C. Z., Sorel, I., Ferrer, J. L., Poupon, A., Janin, J., and van Tilburgh, H. (2004) Proteins 54, 776–783
30. Ryu, K. S., Kim, C., Park, C., and Choi, B. S. (2004) J. Am. Chem. Soc. 126, 9180–9181
31. Meyer, B., and Peters, T. (2003) Angew Chem. Int. Ed Engl. 42, 864–890
32. Ryu, K. S., Kim, C., Kim, I., Yoo, S., Choi, B. S., and Park, C. (2004) J. Biol. Chem. 279, 25544–25548
33. Howard, S. M., and Heinrich, M. R. (1965) Arch. Biochem. Biophys. 110, 395–400
34. Pauly, H. E., and Pfleiderer, G. (1975) Hoppe Seylers Z. Physiol. Chem. 356, 1613–1623
35. Livingstone, G., Franks, F., and Aspinall, L. J. (1977) J. Sol. Chem. 6, 203–216
36. Frey, P. A. (1996) *Faseb J.* **10**, 461–470
37. Petry, K. G., and Reichardt, J. K. (1998) *Trends Genet.* **14**, 98–102
38. Novelli, G., and Reichardt, J. K. (2000) *Mol. Genet. Metab.* **71**, 62–65
39. Assairi, L., Bertrand, T., Ferdinand, I., Slavova-Azmanova, N., Cristensen, M., Briozzo, P., Schaeffer, F., Craescu, C. T., Neuhard, J., Barzu, O., and Gilles, A. M. (2004) *Protein Sci.* **13**, 1295–1303
40. Ryu, K. S., Kim, J. I., Cho, S. J., Park, D., Park, C., Cheong, H. K., Lee, J. O., and Choi, B. S. (2005) *J. Mol. Biol.* **349**, 153–162
41. Rose, I. A., O’Connell, E. L., and Schray, K. J. (1973) *J. Biol. Chem.* **248**, 2232–2234
42. Nogae, I., and Johnston, M. (1990) *Gene (Amst.)* **96**, 161–169
43. Salas, M., Vinuela, E., and Sols, A. (1965) *J. Biol. Chem.* **240**, 561–568