Crystal Structure of Hexokinase KlHxk1 of Kluyveromyces lactis

A MOLECULAR BASIS FOR UNDERSTANDING THE CONTROL OF YEAST HEXOKINASE FUNCTIONS VIA COVALENT MODIFICATION AND OLIGOMERIZATION

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Crystal structures of the unique hexokinase KlHxk1 of the yeast Kluyveromyces lactis were determined using eight independent crystal forms. In five crystal forms, a symmetrical ring-shaped homodimer was observed, corresponding to the physiological dimer existing in solution as shown by small-angle x-ray scattering. The dimer has a head-to-tail arrangement such that the small domain of one subunit interacts with the large domain of the other subunit. Dimer formation requires favorable interactions of the 15 N-terminal amino acids that are part of the large domain with amino acids of the small domain of the opposite subunit. The head-to-tail arrangement involving both domains of the two KlHxk1 subunits is appropriate to explain the reduced activity of the homodimer as compared with the monomeric enzyme and the influence of substrates and products on dimer formation and dissociation. In particular, the structure of the symmetrical KlHxk1 dimer serves to explain why phosphorylation of conserved residue Ser-15 may cause electrostatic repulsions with nearby negatively charged residues of the adjacent subunit, thereby inducing a dissociation of the homologous dimeric hexokinases KlHxk1 and ScHxk2. Two complex structures of KlHxk1 with bound glucose provide a molecular model of substrate binding to the open conformation and the subsequent classical domain closure motion of yeast hexokinases. The entirety of the novel data extends the current concept of glucose signaling in yeast and complements the induced-fit model by integrating the events of N-terminal phosphorylation and dissociation of homodimeric yeast hexokinases.

The enzymes of the hexokinase family catalyze the intracellular trapping and the initiation of metabolism of glucose, fructose, and mannose. In addition to their role in glycolysis, an increasing number of yeast, plant, and mammalian hexokinases have been found to represent multifunctional proteins that are implicated in glucose sensing and signaling (1–4), whereas their glycolytic sugar substrate plays a dual role as a carbon source and hormone-like regulator (4, 5). The molecular basis underlying the involvement of hexokinases in the transcriptional control of glucose metabolism and in glucose homeostasis is their ability to interact with mitochondria and to reversibly translocate to nuclei (3, 6–9).

In the Crabtree-positive yeast Saccharomyces cerevisiae, glucose abundance is accompanied by the translocation of the cytosolic hexokinase isozyme 2 (ScHxk2) 4 and the transcriptional repressor Mig1 (ScMig1) into the nucleus, where both proteins participate in the formation of a hetero-oligomeric complex that suppresses the transcription of ScMig1 target genes like SUC2 encoding invertase (9). The mechanism of glucose signaling in glucose-repressible strains of the Crabtree-negative yeast Kluyveromyces lactis, used increasingly as a model organism in comparative functional genomics (10–12), is largely unknown; however, the unique hexokinase KlHxk1 encoded by the RAGS gene, the expression of glucose transporters, and the capacity for glucose transport seem to be involved (13–15).

Contrary to the situation in bakers’ yeast, glucose and fructose limitation causes the translocation of the mammalian hexokinase isozyme IV (also referred to as “glucokinase” or hexokinase D) to the nucleus of the liver parenchymal cell where it binds to its regulatory protein, GKR, and retranslocates when glucose is abundantly available again (16). The mechanism by which glucokinase contributes to glucose-sens-

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4 The abbreviations used are: ScHxk2, S. cerevisiae hexokinase isozyme 2 (B or PI); KlHxk1, K. lactis hexokinase 1; ScHxk1, S. cerevisiae hexokinase isozyme 1 (A or PII); SmHxk1, S. mansoni hexokinase 1; KlMig1, K. lactis Mig1 regulatory protein; ScMig1, S. cerevisiae Mig1 regulatory protein; PDB, Protein Data Bank; SAXS, small-angle x-ray scattering; Bicine, N,N-bis(2-hydroxyethyl)glycine; CHES, 2-(cyclohexylamino)ethanesulfonic acid; AMP-PCP, adenosine 5’-bis(β,γ-methylene)triphosphate; AMP-PNP, adenosine 5’-β,γ-imino)triphosphate.
Crystal Structure of KlHxk1

ing and regulation of insulin secretion in the pancreatic β-cell is understood only in part at present (17). Despite steadily increasing evidence for multiple hexokinase-protein interactions and translocations to subcellular structures in yeast (9) and mammals (17), the exploration of the structural basis enabling hexokinases to fulfill their respective functions remains a challenging task.

The carbon source-dependent nucleocytoplasmic translocation of ScHxk2 in the fermentative yeast S. cerevisiae has been studied in detail using genetic, immunological, and microscopic approaches (9). Because of the observation that glucose limitation stimulates both the phosphorylation of ScHxk2 and the dissociation of the homodimeric enzyme (20), and its export from the nucleus (9), the covalent modification at Ser-15 and subsequent changes of the oligomeric state have been presumed to be involved in the control of ScHxk2 regulatory functions (9, 21). In contrast, no data are available on the covalent modification and/or intracellular distribution of KlHxk1 in the nonfermentative yeast K. lactis. The two hexokinases exhibit 73% sequence identity (22), and dimerization of the K. lactis enzyme apparently decreases its glucose kinase activity (23), as observed similarly for ScHxk2 (21). In addition, the monomer-dimer equilibrium of both hexokinases depends in a comparable manner on the enzyme concentration and on the presence of substrates and products with glucose most strongly inducing homodimer dissociation (21, 23).

To explore the interrelation between the structure and function(s) of hexokinases, crystal structures of isoenzymes ScHxk1 (hexokinase A or PI) and ScHxk2 (hexokinase B or PII) from the eukaryotic model organism S. cerevisiae have been determined, which have rendered these enzymes a classical example of substrate-induced domain closure motion in enzyme catalysis (24–27). The molecular bases of the description of this closure motion are the structures of ScHxk1 in complex with glucose (PDB entry, 1HKG; the glucose molecule is not present in the PDB file (25)) and of the substrate-free ScHxk2 isoenzyme (PDB entry, 2YHX) (28). Recently, structures have been determined to higher resolution for the two proteins. In the case of ScHxk1, the same crystal form was analyzed (PDB entry, 3B8A) (29), whereas a new crystal form was obtained for ScHxk2 (PDB entry, 11G8) (30). Remarkably, in all of these structures the enzymes are present in the monomeric state, even though ScHxk1 and ScHxk2 are known to form dimers (31). In contrast, dimeric arrangements have been observed in crystal forms BI (32) and BII (33) of ScHxk2; however, these dimers are asymmetric and consist of two subunits related by screw axes of 156° (BI) and 180° (BII) rotation and 13.8 Å (BI) and 3.6 Å (BII) translation. Their calculated radii of gyration are 40.2 Å (BI) and 31.3 Å (BII). Only the radius of the latter crystal dimer is in agreement with the radius of gyration of 30–31.3 Å determined from small-angle x-ray scattering of dimeric ScHxk2 in solution (34). It remains unclear whether the asymmetric dimer in crystal form BII corresponds to the dimer observed in solution, as oligomers usually exhibit a closed symmetry.

The present article describes for the first time a symmetric homodimeric yeast hexokinase structure as determined for full-length KlHxk1 by x-ray crystallography using several crystal forms. Small-angle x-ray scattering of the enzyme in solution confirmed the dimer structure observed in the KlHxk1 crystals. Taking into consideration the high degree of sequence identity and the similar oligomerization behavior of KlHxk1 and ScHxk2, as well as the detection of KlHxk1 phosphorylation at Ser-15 in preliminary studies, the novel structural model seems appropriate to explain the regulation of KlHxk1 and, by analogy, of ScHxk2 function(s) by covalent modification and modulation of the monomer–dimer equilibrium. In addition, the crystal structure analysis of open and closed forms of KlHxk1 including enzyme-glucose complexes as presented here allows for a re-evaluation of the induced-fit model of yeast hexokinase-glucose interaction using the same isoenzyme as the object of study.

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Analysis—KlHxk1 was prepared as described previously (23). A final gel filtration chromatography step was performed using a Superdex 200 16/60 pg column (GE Healthcare) equilibrated with 10 mM Tris-HCl buffer containing 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.4 (chromatography buffer), at a flow rate of 1 ml/min. Protein concentrations were determined according to Bradford (35) using bovine serum albumin fraction V (lot 3X04340, Applichem, Darmstadt, Germany) as a standard. Details of the preparation procedure and analytics of Ser-15-phosphorylated KlHxk1 will be described elsewhere.

Briefly, the Ser-15-phosphorylated enzyme was isolated from K. lactis strain JA6Δrag5R transformed with multicopy vector pTSRAG5 carrying the RAG5 gene and the selection marker URA3 (23). Cells were grown in yeast nitrogen base (YNB)/dextrose medium lacking uracil and supplemented with amino acids according to Sherman et al. (46) at pH 5.5 and 30 °C until an A 600 of 2 was reached. The initial concentration of glucose was 2%. The isolated enzymes were subjected to electrospray ionization mass spectrometry to verify their molecular identity and native state (see supplemental Fig. 2) and, in the case of phosphorylated KlHxk1, to check phosphorylation at Ser-15.

Crystalization, Data Collection, and Data Processing—KlHxk1 was crystallized in different crystal forms. The crystallographic parameters of several crystal forms were described previously (36). For eight crystal forms, full data sets were collected, and the crystal structures were refined (Table 1). All crystals were grown at 291 K starting from an initial concentration of unphosphorylated KlHxk1 of 10 mg/ml. In the case of crystal form XI, consisting of Ser-15-phosphorylated KlHxk1, the initial protein concentration was 7.5 mg/ml. Further details of the crystalization conditions are listed in Table 1. For cryoprotection, crystals were transferred to dried parafin oil (37) or to a buffer containing the reservoir solution and up to 20% glycerol. The glycerol concentration was increased stepwise 5% at a time with a soaking time of about 30 s. Alternatively, the crystals were plunged directly into liquid nitro-

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Crystal Structure of KlHxk1

The crystal structure of KlHxk1 was analyzed in eight different crystal forms (Table 1). One prominent result is the detection of symmetric ring-shaped homodimers as occurring in crystal forms I–IV and VII with dimensions of about 103 × 81 × 55 Å (Table 2, supplemental Table 1, and Fig. 1A). The dimeric structures of KlHxk1, depicted in supplemental Table 1, lines 2–10, are considered unphysiological dimers generated by crystal packing, as outlined under “Discussion.” In crystal forms VIII, IX, and XI, the enzyme is present as a monomer (Table 2). The ring-shaped dimer exhibits almost perfect C2 symmetry, e.g. a rotation angle of close to 180° and a translation component of less than 0.3 Å, although the dimer axis is a crystallographic 2-fold axis and the C2 symmetry is perfect in crystal form II only. In crystal form I, the orientation of the subunits of the ring-shaped dimer deviates by up to 10.9° from the subunit orientation in the respective ring-shaped dimers of the other crystal forms. This deviation also affects the C2 symmetry as reflected by a rotation angle of 178.8° and a translation component of 3.0 Å.

To distinguish between physiological and artificial oligomeric assemblies, the contact area of all of the observed dimeric structures of KlHxk1 was calculated. The ring-shaped dimer has a contact area of ~1860 Å². This number significantly exceeds the threshold value of 600 Å² above which physiologically relevant dimers are to be expected (54). The other putative dimers in the crystal packing (Table 2) have significantly lower contact areas, not exceeding 659 Å². It should be noted that an asymmetric dimer similar to the one found in Schxk2 crystal forms BII (32) and BII (33) is not present in the KlHxk1 crystals.

Each KlHxk1 monomer has a ribonuclease H-fold consisting of two domains: a large domain with a central mixed

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**RESULTS**

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TABLE 1
Crystallization, data collection, and refinement statistics

| Crystallization, data collection, and refinement statistics | Crystal form |
|-----------------------------------------------------------|-------------|
| X-ray source                                              | BESSY II 14.3 |
| Wavelength (Å)                                            | 0.894       |
| Temperature (K)                                           | 100         |
| Cryoprotection                                           | Paraffin oil |
| Processing program                                       | XDS         |
| Crystallization additive                                  | H2O         |
| Unit cell size                                            | 14.50       |
| Unit cell (Å)                                             | 98.19       |
| Resolution, last shell (Å)                                | 2.05–2.00   |
| Completeness (%)                                          | 99.8 (98.9) |
| Unique reflections                                       | 69,639      |
| Multiplicity                                             | 14.6 (1.5)  |
| Rsym (%)                                                  | 7.0 (50.5)  |
| Rfree (%)                                                 | 7.7 (55.0)  |
| Average θ(ε)/σ(θ)                                        | 22.0 (4.9) |
| Wilson B (Å²)                                            | 31.3        |
| Polypeptides/asymmetric unit                             | 2           |
| No. amino acids                                           | 950         |
| No. glucose                                               | 4           |
| No. AMP-PNP                                               | 1           |
| No. CHES                                                  | 4           |
| No. phosphates                                           | 23          |
| No. sulfates                                              | 6           |
| No. chloride                                              | 4           |
| No. waters                                                | 400         |
| Rwork/Rfree (%)                                           | 18.5/24.4   |
| r.m.s.d. (Å)                                              | 0.024/0.017 |
| Reference                                                |            |

**v** Crystal form XI was obtained with Ser-15-phosphorylated KlHxk1, whereas all other crystals were grown from the unphosphorylated enzyme.

**v** Prior to data collection, an equal volume of 100 mM AMP-PNP solution was added to the crystallization drop and the sample incubated for 3 days.

**v** Crystal form XI was obtained with Ser-15-phosphorylated KlHxk1, whereas all other crystals were grown from the unphosphorylated enzyme.

**v** Interdomain rotation angle calculated relative to KlHxk1 crystal form XI representing the most closed KlHxk1 conformer.

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**v** Glucose is not present in the PDB file; its binding is described in the quoted reference.

**v** Ortho-toloylglucosamine.

**v** Phosphoserine 15-KlHxk1.

- **β**-sheet (strands 1, 11, 10, 9, 12, and 13) and a smaller domain with strands 6, 7, 5, 2, 3, and 4 as the core structures (Fig. 1, B and C). The **β**-sheets are flanked by α-helices. In the dimer, the small domain interacts with the large domain of the opposite monomer in a head-to-tail arrangement (Fig. 1, A and D).

TABLE 2
Comparison of yeast hexokinase crystal structures

| PDB entry | KhHxk1 crystal form | Protein | Oligomeric state | Chain | Residues | Angle (°) |
|-----------|----------------------|---------|-----------------|-------|----------|-----------|
| 1HKG      | ScHxk1               | (Glucose)*[^a^] | Monomer | A     | 19–485   | 3.2 (6.0) |
| 3B8A      | ScHxk1               | Glucose, SO₄²⁻ | Monomer | A     | 15–147, 149–485 | 0.0 (4.9) |
| 2YHX      | ScHxk2               | OTG⁺ | Monomer | A     | 19–485   | 15.3 (17.2) |
| 1IG8      | ScHxk2               | SO₄⁻ | Monomer | A     | 18–485   | 17.8 (19.3) |
| 3O08      | KlHxk1               | SO₄⁻ | Dimer | A     | 15–485   | 14.6 (17.6) |
| 3O1B      | KlHxk1               | Glyceral, PO₄³⁻ | Dimer | A     | 9–485    | 15.8 (18.2) |
| 3O1W      | KlHxk1               | Glyceral, PO₄³⁻ | Dimer | A     | 2–485    | 18.9 (21.1) |
| 3O4W      | KlHxk1               | Glyceral, SO₄²⁻ | Dimer | A     | 2–485    | 18.9 (21.1) |
| 3O5B      | KlHxk1               | Glyceral, SO₄²⁻ | Dimer | A     | 7–485    | 17.7 (20.3) |
| 3O6W      | KlHxk1               | Glyceral, PO₄³⁻ | Monomer | A     | 19–485   | 21.9 (24.2) |
| 3O80      | KlHxk1               | AMP, PO₄³⁻ | Monomer | A     | 16–485   | 25.8 (28.1) |
| 3O8M      | KlHxk1 (Ser-15-P)    | Glucose | Monomer | A     | 15–485   | 4.9 (0.0) |

*[^a^] Ligands bound to the hexose or ATP binding sites.

*[^b^] Interdomain rotation angle calculated relative to ScHxk1 crystal structure 3B8A (29), the most closed S. cerevisiae hexokinase conformer.

*[^c^] Added 10 mM glucose to crystallization solution.

*[^d^] No. waters calculated after scaling.

*[^e^] Added 2m M AMP-PCP to crystallization solution.

*[^f^] Added 10 mM MgCl₂ to crystallization solution.
FIGURE 1. **Crystal structure of dimeric KlHxk1.** A, stereoview of homodimeric KlHxk1. Monomer A is shown with blue β-strands and red helices for the large domain and violet β-strands and yellow helices for the small domain. The N terminus (residues 2–20) is shown in green. B, monomer structure with labeled α-helices (A–O) and β-strands (1–13). C, topology diagram of KlHxk1 with residue numbers. The circles and triangles correspond to α-helices and β-strands, respectively. D, interactions of the N terminus of chain A (green) with amino acids of the adjacent monomer (gray) within a 4 Å distance. Side chain carbon atoms are colored green for chain A and yellow for chain B. Hydrogen bonds (≤3.5 Å) are depicted as dotted lines.
Solution Structure of KlHxk1 — To identify the physiological dimer structure of KlHxk1, SAXS data of the unphosphorylated enzyme were collected. The experimental radius of gyration of KlHxk1 of 32.7 Å determined from the SAXS data closely corresponds to the value of 32.2 Å calculated for the ring-shaped dimer. Further, the scattering curves calculated from the ring-shaped dimer forms of KlHxk1 (Fig. 2A) provide good fits to the experimental scattering curves with discrepancy values between 1.37 and 1.62 (listed in supplemental Table 1). This clearly indicates that the ring-shaped dimer exists also in solution at protein concentrations higher than 3.5 mg/ml. A rigid body refinement of the two subunits of the crystal form IV dimer toward the SAXS data improves even further to 1.31 while keeping the same ring-shaped arrangement of the dimer. In contrast, the $\chi^2$ value of the KlHxk1 monomer calculated for chain A of crystal form III is very large (14.2), and the computed pattern displays significant deviations from the experiment (Fig. 2A). The remaining putative dimer structures depicted in supplemental Table 1 are characterized by significantly worse fits of their calculated scattering curves to the SAXS curves, giving $\chi^2$ values between 4.03 and 13.85.

Based on the SAXS data, an envelope model of KlHxk1 was computed independently by ab initio shape reconstruction imposing no symmetry (Fig. 2B). The resulting model is in fairly good agreement with the ring-shaped dimer. Minor differences between the envelope shape and the crystallographic model (e.g. the pore in the crystallographic dimer, which is not present in the envelope structure) may be attributed to the molecular flexibility of the enzyme in solution and/or the lower resolution of SAXS, which precludes a more precise shape determination.

Structure and Role of the N Terminus of KlHxk1 — The conformation of the N-terminal residues of KlHxk1, SAXS data of the unphosphorylated enzyme were collected. The experimental radius of gyration of KlHxk1 of 32.7 Å determined from the SAXS data closely corresponds to the value of 32.2 Å calculated for the ring-shaped dimer. Further, the scattering curves calculated from the ring-shaped dimer forms of KlHxk1 (Fig. 2A) provide good fits to the experimental scattering curves with discrepancy $\chi^2$ values between 1.37 and 1.62 (listed in supplemental Table 1). This clearly indicates that the ring-shaped dimer exists also in solution at protein concentrations higher than 3.5 mg/ml. A rigid body refinement of the two subunits of the crystal form IV dimer toward the SAXS data improves even further to 1.31 while keeping the same ring-shaped arrangement of the dimer. In contrast, the $\chi^2$ value of the KlHxk1 monomer calculated for chain A of crystal form III is very large (14.2), and the computed pattern displays significant deviations from the experiment (Fig. 2A). The remaining putative dimer structures depicted in supplemental Table 1 are characterized by significantly worse fits of their calculated scattering curves to the SAXS curves, giving $\chi^2$ values between 4.03 and 13.85.

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Structure and Role of the N Terminus of KlHxk1 — The conformation of the N-terminal residues of KlHxk1 is defined to a different extent in our crystals (Table 2). In crystal forms III and IV, residues 2–485 are ordered well enough to allow the building of a complete subunit model (supplemental Fig. 1). Residue Met-1 is not present in the native KlHxk1 protein (23). The N terminus adopts an extended conformation and folds along the dimer interface into the cleft between both domains of the opposing subunit (Fig. 1A). Remarkably, the calculated buried surface area is reduced from 3720 to 780 Å² when residues 2–20 are omitted from the structure of the KlHxk1 dimer. This considerable difference illustrates the
important role that the N terminus plays in dimer formation. In crystal form III, the average B-value (temperature factor) of the 10 N-terminal residues is 40 Å² compared with 22 Å² for the rest of the protein, which indicates a higher mobility of the N terminus in the crystal as well. The latter finding is in agreement with the disorder of N-terminal amino acids observed in the other crystals including the dimeric structures (Table 2). In crystal forms VIII, IX, and XI containing monomeric enzyme, even more of the amino acids of the N terminus are flexible (Table 2), as indicated by their absence from the electron density map. The assumption that disorder and not absence due to proteolysis causes the limited visibility of N-terminal residues in the electron density maps is based on mass spectrometry data (not shown), proving the integrity of all KlHxk1 preparations used for crystallization in the present study.

**Ligand Binding to KlHxk1**—Depending on the composition of the crystallization reservoir solution and the presence of additives during crystallization and cryoprotection, low molecular weight compounds (glucose, glycerol, AMP, sulfate, and phosphate) are found at the hexose or ATP binding sites in some of the KlHxk1 crystals (Table 2). In crystal forms VII and XI containing KlHxk1 in an open and closed state, respectively, a glucose molecule is complexed by hydrogen bonds to the side chains of Asn-209, Asp-210, Thr-211, Glu-268, and Glu-301 and to the main chain amide of Gly-234 (Fig. 3). In the closed state, two loops of the small domain are involved in additional interactions with glucose via hydrogen bonds to the side chains of Thr-233 and Ser-418 side chains of the open state structure. This binding site presumably interacts with the α-phosphate of ATP, as indicated by a superposition of ADP from an ADP-glucose complex structure of human hexokinase (PDB entry 1DGK) (55) (Fig. 3B).

In crystal form IX, grown in the presence of AMP-PCP and soaked with 50 mM AMP-PNP, the nucleotide analog is bound to the active site of KlHxk1 at two positions; however, only the AMP moiety is defined in the electron density maps. One AMP group is oriented in a way that its phosphate tail is not pointing toward the glucose binding site, whereas another AMP group is bound close to residues Thr-35, Ser-38, Thr-389, and Arg-393 (data not shown). Interestingly, these amino acids correspond to the regulatory ATP binding site of human hexokinase I (55). The observed binding of AMP-PNP in different orientations to KlHxk1 and the lack of evidence for a regulatory ATP binding site in yeast hexokinases may indicate that this observation is an artifact attributable to the high AMP-PNP concentration of the crystal-soaking buffer.

**Domain Flexibility of Yeast Hexokinases**—Table 2 summarizes the interdomain rotation angles of the presently known structures of yeast hexokinases relative to the closed structures of KlHxk1 and ScHxk1 (PDB entry 3B8A) (29). The largest difference in the relative orientation of the two domains of 28.1° is observed when the KlHxk1 enzymes in crystal forms IX and XI are compared (Table 2 and Fig. 4).
form XI corresponds to the most closed state, whereas the enzyme in crystal form IX exhibits the most open conformation. The 28.1° interdomain rotation axis, describing the relative reorientation of the two domains between the most open and most closed form of KlHxk1, aligns well with the interdomain rotation axis describing the 17.8° domain movement between the open state of ScHxk2 and the closed conformation of ScHxk1 (Fig. 4A). It should be noted, however, that the latter two structures do not necessarily reflect a substrate-induced conformational transition because they belong to

FIGURE 4. Comparison of the domain flexibility of yeast hexokinases KlHxk1, ScHxk1 and ScHxk2 (stereoview). The structures have been superimposed based on the Cα atoms of the large domains (shown in the upper part of the images). A, superposition of KlHxk1 crystal forms XI (green) and IX (blue) and ScHxk1 (yellow) (29) and ScHxk2 (black) (30). The interdomain rotation axes of KlHxk1 crystal form IX and of ScHxk2 are calculated relative to KlHxk1 crystal form XI or ScHxk1, respectively, using the same color code. Glucose molecules from the structures of ScHxk1 (29) and of KlHxk1 crystal form XI are shown in yellow and green, respectively. B, domain interfaces of KlHxk1 crystal form XI (green) and ScHxk1 (yellow) (29) with selected residues shown as sticks. The configurations of the glucose molecules are labeled at their 1-OH positions. Polar interactions are shown as dotted lines.
different isoenzymes of *S. cerevisiae* hexokinase (29, 30), which share 77% sequence identity (22).

The determination of the open and closed forms of one and the same yeast hexokinase as described for the first time in the present study and the significantly larger interdomain rotation angle observed for KlHxk1 suggested a re-evaluation of the domain closure motion of yeast hexokinases. For an unbiased determination of the dynamic domains, the program DynDom (56, 57) was used (Fig. 5). Residues that move together as a rigid body during the conformational change also cluster close together in the diagram depicting the rotation vectors as points in a Cartesian coordinate system (Fig. 5, B and D). This analysis, based on the most open (crystal form IX) and closed states (crystal form XI) of KlHxk1, shows that three dynamic domains can be distinguished. The large domain and parts of the small domain move relative to each other to open and close the active site (Fig. 5A), but they also move relative to helices E and O and nearby residues, which thus form a third, central domain. In the movies provided as supplemental material, the central domain is held fixed, *i.e.* the open and closed structures are superimposed based on these residues, for the animation. The movement of both do-

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**FIGURE 5. Determination of the dynamic domains of KlHxk1 by comparison of the most open (crystal form IX) and the most closed (crystal form XI) conformers.**

A, description of domain rotation by three dynamic domains (stereoview). The small (red) and large (blue) domains move relative to the central domain (yellow). The rotation axes are indicated in red. In all panels, the bending residues are shown in green. B, cluster analysis of the rotation vectors for the three-domain description. C, alternative description of KlHxk1 domain rotation based on two dynamic domains (stereoview). The view is along the rotation axes, indicated in red. D, cluster analysis of the rotation vectors for the two-domain description.
mains relative to the central domain is mediated by an interface consisting of a β-sheet of the small or large domain, respectively, and an α-helix of the central domain. At both interfaces, the rotation axis lies approximately in the middle of the curved β-sheet plane (perpendicular to the β-strands). Thus, the β-sheet rotates around the α-helix. However, whereas the β-sheet of the larger domain rotates almost as a rigid body, the β-sheet of the smaller domain is bent with the rotational domain movement and increases in curvature (Fig. 5 and supplemental Movie 1). The screw operator describing the movement of the small dynamic domain relative to the central domain has a rotation angle of 14.9° and a translation of 0.2 Å, whereas the screw operator of the movement of the large domain relative to the central domain has an 18.4° rotation and a 0.3 Å translation. Both axes describe an almost pure closure motion with values of 92.3 and 99.0%.

In an alternative description, two domains are found in the DynDom analysis (Fig. 5C and supplemental Movie 2). This description was obtained from slightly different starting structures, e.g. minor differences in the coordinates or differences in the orientation of the proteins used for the DynDom analysis. Thus, the two-domain and three-domain descriptions almost equally satisfy the criteria for determining dynamic domains by the clustering algorithm implemented in DynDom. The residues of the central domain of the three-domain description now belong mostly to the larger domain (depicted in blue in Fig. 5C). The 27.0° rotation axis (translation component 0.03 Å), which is located between helices E and O, describes a classical hinge-bending domain movement with 99.8% closure motion. Both descriptions are equally suitable to characterize the domain movement of hexokinase KlHxk1. The two-domain model of the hexokinase closure motion exhibits the advantage of being simpler and is in agreement with previous descriptions (58) obtained for a much smaller (13°) hexokinase domain motion. However, the cluster analyses shown in Fig. 5, B and D, indicate that not all residues of the large and small domain move as a rigid body. In particular, the residues around helices E and O deviate in that their rotation vectors are in between those of the large and small domains. These residues are thus clustered to form a third (central) domain relative to which the small and large domains move during the conformational transition between the open and closed states. The more detailed picture of the molecular mechanism of the hexokinase domain movement presented here is also supported by the interpolated morphs that visualize the above conformational transition of KlHxk1 (supplemental Movies 1 and 2).

**Comparison of Hexokinase Structures**—The closest structural homologs of KlHxk1 are the hexokinases ScHxk1 (29) and ScHxk2 (30) from *S. cerevisiae* exhibiting about 70 and 73% sequence identity, respectively, with the *K. lactis* enzyme. The residues involved in KlHxk1 dimerization via hydrogen bonding interactions are conserved in the *S. cerevisiae* hexokinases with two exceptions: the replacement of Asn-110 by serine in ScHxk1 and ScHxk2 and the conservative substitution of Arg-113 by lysine in ScHxk1 (Fig. 1D). Likewise, the residues forming hydrophobic intersubunit interactions are largely conserved, with only Pro-10 (Gln-10 in ScHxk1 and ScHxk2) and Trp-141 (Gln-142 in ScHxk1 and ScHxk2) being exchanged. In total, more than 84% of the residues forming the KlHxk1 dimer interface are conserved in ScHxk1 and ScHxk2.

Interestingly, a systematic search in the RCSB Protein Data Bank for dimeric sugar kinases that are structurally related to KlHxk1 resulted in a single entity: hexokinase SmHxk1 of the rather distantly related worm *Schistosoma mansoni*. This enzyme, although exhibiting only 33% sequence identity (59), forms a similar ring-shaped dimer in the crystal (Fig. 6) with a contact area of 1330 Å², but occurs in a monomeric state in solution (60). As the dimer contact area of SmHxk1 is smaller...
than that of KlHxk1 by only about 500 Å², the worm hexokinase may dimerize at the high protein concentration existing in the crystal but may dissociate at the significantly lower protein concentrations typically employed in solution studies.

**DISCUSSION**

**Crystal and Solution Structures of KlHxk1**—The hexokinase KlHxk1 forms a monomer-homodimer equilibrium with the dimeric species predominating at enzyme concentrations above 1 mg/ml under *in vitro* conditions (23). An estimation of the average concentration of the enzyme in the cytosol has shown that dimer formation is likely to occur also in the cell (23). In the crystal, the high protein concentration should strongly favor dimer formation. The observation of a ring-shaped dimer in several crystal forms of KlHxk1 (supplemental Table 1), its closed C₄ symmetry, the large surface area of 3700 Å² buried upon dimer formation, and most importantly, the confirmation of the dimer structure by SAXS data (Fig. 2) suggest the existence of the ring-shaped dimeric enzyme also under physiological conditions. In particular, the specific subunit assembly of the symmetric KlHxk1 dimer provides a structural basis that allows to understand on a molecular level the phenomena that were observed for both *S. cerevisiae* and *K. lactis* hexokinases: the dependence of glucose kinase activity on the enzyme concentration and oligomeric state, respectively (21, 23), the influence of substrates and other ligands on the monomer-dimer equilibrium (21, 23), and the impact of Ser-15 phosphorylation on hexokinase dimerization (20) and putative regulatory functions (9, 21, 23). These phenomena will be discussed in detail in the following paragraphs considering structure-function interrelations.

In crystal form XI, which was grown from Ser-15-phosphorylated KlHxk1, the enzyme is present as a monomer. This finding coincides with preliminary data indicating that Ser-15 phosphorylation induces monomer formation, as discussed below. There are several explanations for KlHxk1 monomers being also observed in crystal forms VIII and IX (Table 2), although they have been grown from the unphosphorylated enzyme. First, crystal packing may affect the monomer-dimer equilibrium. When the monomeric but not the dimeric enzyme fits into the structure of a crystal form, crystal growth will constantly withdraw the monomeric enzyme from the equilibrium, and crystals of the monomer will be formed, provided that a significant concentration of monomeric enzyme is present for crystal nucleation. Second, the monomeric species may be favored by specific crystallization conditions. In detail, crystal form VIII was grown in the presence of the glucose analog D-xylose and ATP, whereas crystal form IX was grown in the presence of nonhydrolyzable ATP analog AMP-PCP. In accordance with the dissociative effect exerted by glucose and glucose 6-phosphate on both KlHxk1 and ScHxk2 (21, 23), the presence of (pseudo)substrates may increase the partial concentration of the monomer. Third, a proteolytic removal of N-terminal amino acids known to stabilize the dimeric structure of the homologous hexokinase ScHxk2 (61) cannot be excluded as a cause for monomer formation during long-term crystallization. The visibility of all residues of the N terminus of KlHxk1 in the electron density of a form III crystal harvested together with a form VIII crystal from the same crystallization droplet 1 year after crystallization setup, however, argues against N-terminal truncation.

The lack of evidence for a symmetric dimeric subunit assembly in the published crystal structures of the hexokinases ScHxk1 and ScHxk2 could be due to crystal packing interactions and specific crystallization conditions as discussed for KlHxk1. In contrast to the situation with KlHxk1, however, the most probable explanation is related to the N terminus, which in the crystal structures of monomeric ScHxk1 (PDB entries, 1HKG (25) and 3B8A (29)) and ScHxk2 (PDB entries, 2YHX (28) and 1IG8 (30)) is not modeled. The latter situation may reflect either a disorder of the respective N-terminal residues or their proteolytic removal. Indeed, ScHxk2 crystal form BIII (PDB entry, 2YHX) (28) was grown from enzyme lacking the N-terminal 11 amino acids (61), whereas N-terminal integrity is unknown in the case of yeast hexokinase structures 1HKG (25), 3B8A (29), and 1IG8 (30).

It remains to be shown whether ScHxk2 forms a similar ring-shaped dimer as KlHxk1 in solution. From SAXS data, a radius of gyration of 31.3 Å was obtained for ScHxk2 (34), which closely corresponds to the calculated radius of gyration of 31.5 Å of the dimer in crystal form BII (33). However, the latter dimer is asymmetric with a rotation angle of 156° and a translation of 13.8 Å. Another asymmetric dimer of ScHxk2 observed in crystal form BII (32), exhibiting a calculated radius of gyration of 40.2 Å, was considered unphysiological (34). The close similarity of the radii of gyration of the symmetric KlHxk1 dimer (32.0 Å) and the asymmetric ScHxk2 dimer (31.3 Å) suggests that both hexokinases might form similar ring-shaped dimeric structures in solution.

**Domain Closure Motion, Ligand Binding, and Catalysis**—The current knowledge of the domain closure motion of yeast hexokinases is based on and limited by the comparison of different isoenzymes of *S. cerevisiae* hexokinase; ScHxk1 is considered to represent the closed state (25, 29), whereas ScHxk2 exhibits a comparatively open conformation (28, 30). In contrast, the structural data presented in the present article were obtained with the single enzyme KlHxk1. The crystal structures of KlHxk1 exhibit variations of the interdomain rotation angle of up to 28.1° (Table 2). KlHxk1 crystal forms I, II, III, IV, VII, VIII, and IX contain the enzyme in open conformational states, whereas crystal form XI represents the only observed closed conformation that is similar to the ScHxk1 structure (PDB entry, 3B8A (29)). Form IX of KlHxk1 exhibits an interdomain rotation angle of 25.8° (as compared with ScHxk1) and is significantly more open than the most open conformer of ScHxk2 (PDB entry, 1IG8 (30)) with an angle of 17.8° (Table 2). The interdomain rotation axis vector of the KlHxk1 domain movement between crystal forms IX and XI coincides closely (16° deviation, Fig. 4A) with the domain closure motion vector of the hexokinases of *S. cerevisiae* as inferred from a comparison of ScHxk2 (30) in the open conformation and ScHxk1 (29) in the closed conformation. This finding indicates that the three homologous hexokinases undergo similar domain movements during their catalytic cycles.

The glucose kinase activities of KlHxk1 and ScHxk2 have been shown to depend on the enzyme concentration, with the
dimer species being apparently less active (21, 23). It is currently unclear whether the dimer is completely inactive and catalytic activity is exclusively associated with the fraction of monomeric enzyme being in equilibrium with the dimer or whether the dimeric hexokinase displays a reduced activity compared with the monomer. In accordance with the findings of the present study, the catalytic activity of KlHxk1 may be reduced or abolished by dimer formation via one of the following scenarios: (i) dimer formation may distort the structure of the active site such that the catalytic activity is affected; (ii) dimer formation may reduce the rate of the conformational change between the open and closed states by increasing the activation barrier of the conformational change; or (iii) dimer formation may affect the equilibrium between the open and closed states by stabilizing either the open or the closed state. Scenario (i) cannot be evaluated at present because no KlHxk1 dimeric structure representing the closed state is available. Concerning scenario (ii), it appears feasible that dimer formation influences the rate of the conformational change because the intersubunit interactions mediated in the ring-shaped dimer by two pairs of non-identical domains of opposite subunits are likely to interfere with the domain closure motion. If the conformational change is rate-limiting, dimer formation may affect catalysis by reducing the rate of the domain closure motion. Along the same lines, the involvement of both domains of the KlHxk1 monomer in the formation of the symmetric homodimer makes it likely that one conformational state exhibits more favorable intersubunit interactions than the other and is thus stabilized (scenario (iii)). The finding that all dimeric KlHxk1 structures described in this study represent open enzyme forms might reflect a stabilization of the open conformation in the dimer (although the number of crystal forms analyzed here may not be large enough to allow statistically significant conclusions). In that case, substrate turnover will be reduced if the catalytic events taking place in the closed state with ATP and sugar substrate bound to the enzyme comprise the rate-limiting step in catalysis.

The idea that dimer formation may stabilize the open form of KlHxk1 could also explain the influence of substrates and/or products on the oligomeric state and, thereby, the catalytic activity of the enzyme. Conditions that stabilize the open conformation should also stabilize the KlHxk1 dimer, whereas conditions promoting a closed conformation should shift the association-dissociation equilibrium toward the monomeric enzyme. Indeed, the results of stopped-flow kinetic measurements performed to study the catalytically reacting KlHxk1 over a wide range of enzyme concentrations suggest facilitation of KlHxk1 dissociation by the glycolytic substrates and/or products but also by glucose or glucose 6-phosphate alone (21). The influence of physiological ligands on the association-dissociation equilibrium of the homologous hexokinase ScHxk2 is very similar (21, 23), with glucose exerting by far the strongest dissociative effect (21).

The binding mode of glucose to the open state of KlHxk1 in crystal form VII is similar to the binding mode of the sugar to the closed conformation of KlHxk1 in crystal form XI (Fig. 3B). Importantly, however, two interactions are missing in the open state complex structure; residues Thr-174 and Lys-175 of the small domain are too distant from the glucose ligand to allow favorable hydrogen bonding interactions. Thus, the KlHxk1-glucose complex in crystal form VII may be interpreted as a structural model for sugar binding to the open form of yeast hexokinases via the large domain and as a physiological intermediate being formed before the enzyme adopts a closed conformation. In accordance with the above hypothesis, Kuser et al. (29) postulate for ScHxk1 the existence of an initial glucose binding mode that precedes the large conformational changes, creating the competent glucose binding site (29). The closure motion does not occur in KlHxk1 crystal form VII because the enzyme is fixed by crystal lattice interactions in the open state. After glucose binding to the large domain in solution, however, the above interactions with Thr-174 and Lys-175 can be formed to shift the equilibrium between the open and closed conformations toward the closed state. The transition between the open and closed states observed in crystal forms VII and XI is illustrated by a morph (supplemental Movie 3).

The KlHxk1-glucose complex in crystal form XI, exhibiting an interdomain rotation angle of 4.9°, is significantly more closed than the ScHxk1-glucose (29) complex (Table 2 and Fig. 4). The interactions between the small and large domain near the glucose binding sites of these enzymes are different around residues Arg-294 in KlHxk1 and Arg-295 in ScHxk1, respectively (Fig. 4B). In KlHxk1 crystal form XI, Arg-294 interacts via bifurcated salt bridges with residues Asp-178 and Glu-183, whereas in ScHxk1 Arg-295 forms only a single hydrogen bond to the main chain amide of Trp-174. The interaction of Arg-294 with Asp-178/Glu-183 may stabilize the more closed conformation of KlHxk1.

Another difference between the closed state yeast hexokinase-glucose complexes is that the α-anomer of D-glucose is bound to KlHxk1, whereas the β-anomer of the sugar is coordinated in the active site of the ScHxk1 enzyme (29). In the 15 structures of sugar kinases complexed with substrates that are deposited in the Protein Data Bank, α- and β-anomers are found in about equal shares. In crystal form VII containing KlHxk1 in the open form, glucose is bound as the β-anomer. Interestingly, both anomers have been demonstrated to bind to ScHxk2, but α-D-glucose is the preferred form for the phosphorylation reaction (62). In the KlHxk1 crystal form XI complex structure, the 1-OH group of the glucose α-anomer is bound via a hydrogen bond to the main chain carbonyl group of Val-135, whereas the 1-OH group of glucose in ScHxk2 interacts with the side chain of Thr-174 (Fig. 4B). It should be noted, however, that a small portion (about 20%) of glucose is bound in the β-anomeric form to KlHxk1 crystal form XI as judged by the electron density maps (data not shown).

**Significance of Ser-15 Phosphorylation to KlHxk1 Dimer Stability**—84% of the residues forming the dimer interface in KlHxk1 are conserved in ScHxk1 and in ScHxk2. This value is higher than the overall sequence identity of KlHxk1 with ScHxk1 and ScHxk2 of 70 and 73%, respectively. Consideration of these numbers, together with the fact that (i) residues involved in protein dimerization are usually surface-exposed
in the dissociated state and (ii) surface-exposed amino acids generally tend to be less conserved than amino acids located in the interior of a protein or in the active site, suggests the formation of similar dimers of the three enzymes. In the case of ScHxk2, the phosphorylation of a single amino acid in the dimer interface (Ser-15) is likely to represent an efficient mechanism enabling the cell to induce the dissociation of the hexokinase homodimer, even at the high enzyme concentrations expected to exist in situ (20, 21), and to facilitate ScHxk2 interaction with the major nuclear export receptor Xpo1 (9). The physiological signal that initiates the covalent modification of residue Ser-15 by a hitherto unidentified protein kinase in S. cerevisiae is glucose limitation (19). Interestingly, it was found only recently that KIHxk1 may be phosphorylated in vivo at the equivalent position Ser-15 and that this modification efficiently stimulates the dissociation of the homodimeric enzyme in vitro,5 as observed similarly for ScHxk2 (20).

The crystal structure of the symmetric ring-shaped KIHxk1 dimer provides the unique possibility of understanding on a molecular basis the regulation of the monomer-dimer equilibrium of the enzyme by covalent modification of residue Ser-15. This amino acid is part of the N terminus that protrudes at the dimer interface and even extends into the adjacent subunit and its active site, nearby Asp-210 (Fig. 1, A and D). Ser-15 resides in immediate proximity to the acidic side chains of residues Asp-18, Asp-106, Glu-140, and Glu-355 and the basic side chains of Lys-13–Asp-106 (Fig. 7) with Asp-106 and Glu-140 belonging to the opposite subunit, respectively. Phosphorylation of Ser-15 is likely to cause an electrostatic repulsion of close-by acidic residues at the dimer interface, in particular of Glu-140 (located at negative dipole of helix C) of the adjacent subunit. In addition, repulsion of Asp-18 and Glu-355 may indirectly destabilize the dimeric assembly by disturbing the interactions to their binding partners, Lys-378 and the backbone amid groups of residues 13 and 14, respectively. Dissociation of the N terminus from its binding site by Ser-15 phosphorylation will also destroy the intermolecular salt bridge, Lys-13–Asp-106 (Fig. 7B), and thereby promote monomer formation. Assuming that a similar structural situation exists in the homologous hexokinase ScHxk2, the extensive monomer formation observed as a consequence of Ser-15 in vivo phosphorylation or in vitro exchange by glutamate (20) is likely due to the introduction of a negative charge in the intersubunit interface of the dimeric enzyme.

**Regulatory Role of Yeast Hexokinases**—The understanding of hexokinase involvement in glucose sensing and signaling in yeast on a molecular level is most advanced in S. cerevisiae. In this yeast, a fraction of the cytosolic hexokinase isoenzyme ScHxk2 enters the nucleus when glucose is abundantly available and maintains the interaction of the simultaneously translocated zinc-finger protein suppressor ScMig1 with its target promoters by preventing Mig1 phosphorylation through the ScSnf1 protein kinase complex (63). As a consequence, glucose-repressible genes are repressed and metabolism is focused on glucose utilization. In contrast, glucose limitation triggers ScSnf1-mediated phosphorylation of ScMig1, which is followed by a disintegration of the ScMig1-DNA repressor complex and subsequent derepression of glucose-repressible genes. This regulatory mechanism may be complemented by integrating the monomer-dimer equilibrium of ScHxk2 (20, 21) and by assuming residue Ser-15 to occupy in the homologous hexokinases KIHxk1 and ScHxk2 functionally equivalent positions with respect to dimer stability according to the following scenario. ScHxk2 phosphorylation by a protein kinase of hitherto unknown identity causes the dissociation of the dimeric enzyme, thereby abolishing its interaction with ScMig1. The latter event results in the exposure and phosphorylation of the ScSnf1 target structures of ScMig1, the subsequent dissociation of phospho-ScMig1 from its target DNA, and translocation of the phosphorylated repressor into the cytosol. In addition, phosphorylation of ScHxk2 at Ser-15 strengthens its interaction with the nuclear
Crystal Structure of KlHxk1

exporter, Xpo1, and thereby facilitates the export of the monomeric phosphoenzyme from the nucleus when alternative carbon sources are available (9).

The respective situation has been much less explored in glucose-repressible strains of K. lactis; however, the existence of an ScMig1 homolog (KMig1) has been inferred from homology considerations, and complementation of a mig1 mutant of S. cerevisiae by the homologous K. lactis gene has shown that the function of Mig1 is conserved in these distantly related yeasts (64). Keeping in mind both the considerably high degree of sequence identity between KlHxk1 and ScHxk2 (73%) (22), with a striking conservation of amino acids in the intersubunit interface including the in vivo phosphorylation site, Ser-15, and the comparably high degree of identity (70.6% in an 85-residues overlap) in the N-terminal part of the KIMig1 and ScMig1 proteins, a mechanism of Mig1-hexokinase interaction similar to that proposed for S. cerevisiae may also be anticipated for K. lactis. Indeed, preliminary studies on immobilized KIMig1 13-mer peptides and isolated KlHxk1 enzyme using anti-KlHxk1 antibodies for enzyme-antibody complex detection suggest complex formation.7 In addition, the ring-shaped structure (Fig. 1A) and, in particular, the dimensions of the hole (14–19 Å) in the center of the symmetric KlHxk1 dimer provoke the vision that the enzyme could almost perfectly surround double-stranded B-DNA (helical diameter 20 Å).

The isolation of KIMig1 and the study of its interactions with unphosphorylated and Ser-15-phosphorylated KlHxk1 in the presence of KIMig1-targeted DNA fragments of K. lactis by means of biomolecular interaction analysis and co-crystalization seem to represent a promising approach to further unraveling the regulatory functions of the unique hexokinase, KlHxk1, in the glucose metabolism of K. lactis.

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