Crystal structure of the ADP-dependent glucokinase from *Pyrococcus horikoshii* at 2.0-Å resolution: A large conformational change in ADP-dependent glucokinase

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Abstract

Although ATP is the most common phosphoryl group donor for kinases, some kinases from certain hyperthermophilic archaea such as *Pyrococcus horikoshii* and *Thermococcus litoralis* use ADP as the phosphoryl donor. Those are ADP-dependent glucokinases (ADPGK) and phosphofructokinases in their glycolytic pathway. Here, we succeeded in gene cloning the ADPGK from *P. horikoshii* OT3 (phGK) in *Escherichia coli*, and in easy preparation of the enzyme, crystallization, and the structure determination of the apo enzyme. Recently, the three-dimensional structure of the ADPGK from *T. litoralis* (tlGK) in a complex with ADP was reported. The overall structure of two homologous enzymes (56.7%) was basically similar: This means that they consisted of large α/β -domains and small domains. However, a marked adjustment of the two domains, which is a 10-Å translation and a 20° rotation from the conserved GG sequence located at the center of the hinge, was observed between the apo-phGK and ADP-tlGK structures. The ADP-binding loop (430–439) was disordered in the apo form. It is suggested that a large conformational change takes place during the enzymatic reaction.

Keywords: Crystal structure; ADP-dependent glucokinase; *P. horikoshii*; conformational change; induced fit

Hyperthermophiles are a group of microorganisms that show optimum growth temperatures >80°C (Stetter et al. 1990; Adams 1993). Almost all of them are classified as archaea, the third domain of life (Woese et al. 1990), and recent studies have revealed that these hyperthermophilic archaea have novel sugar metabolic pathways and novel enzymes such as ADP-dependent glucokinase (ADPGK) and ADP-dependent phosphofructokinase (ADPPFK; Schoenheit and Schaefer 1995; Selig et al. 1997).

A novel sugar kinase, ADPGK, was first discovered in the hyperthermophilic archaeon *Pyrococcus furiosus* and was characterized (Kengen et al. 1994, 1995). The enzyme requires ADP as the phosphoryl group donor instead of ATP and is involved in a modified Embden-Meyerhof pathway in this organism (Schoenheit and Schaefer 1995; Selig et al. 1997). Recently, the genes of ADPGK from *P. furiosus* and *Thermococcus litoralis* were cloned, and the products were characterized (Koga et al. 2000). In a phylogenetic tree of the hexokinase family constructed by comparing 60 sequences of sugar kinases, Bork et al. (1993) categorized them in the following three families: (1) hexokinase, (2) ribokinase (RK), and (3) galactokinase. The RK family comprises pro- and eukaryotic RKs, bacterial fructokinases, and *Escherichia coli* 6-phosphofructokinase 2, 6-phosphotagatokinase, 1-phosphofructokinase, and inosine-guano-

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sine kinase. The three-dimensional structures of enzymes belonging to the RK family such as *E. coli* RK (Sigrell et al. 1998), human adenosine kinase (humAK; Matthews et al. 1998), and *Toxoplasma gondii* adenosine kinase (tgAK; Schumacher et al. 2000) have been reported. Recently, the structure of tIGK with ADP (ADP-tIGK), which shares 56.7% sequence homology with ADPGK from *Pyrococcus horikoshii* OT3 (phGK; Fig. 1), was revealed, and it was shown that the topology of tIGK is basically similar to those of ATP-dependent RK and AK (Ito et al. 2001), despite the lack of apparent sequence homology. In ATP-dependent RK and AK, a large conformational change between the apo and holo structures has been observed (Sigrell et al. 1999; Schumacher et al. 2000). However, the structure of apo-

tIGK does not undergo a large conformational change compared with that of ADP-tIGK using the apo crystal, which is prepared by soaking a holo crystal in ADP-free solution (Ito et al. 2001). Thus, it has not been clear whether an induced fit is needed for the enzymatic reaction of ADPGK. Recently, it was found that an enzyme from *Methanococcus jannaschii* showed a high ADP-dependent activity for both glucokinase and phosphofructokinase (Sakuraba et al. 2002). To clarify the enzymatic mechanism of the ADPGK family including ADPFK, we have cloned, expressed, and determined the crystal structure of apo-phGK at 2.0-Å resolution. We found large conformational changes between apo-phGK and ADP-tIGK, indicating the catalytic reaction of glucokinase.

Fig. 1. Structure-based sequence alignment of ADP-dependent glucokinase (ADPGK). Sequences of three ADPGKs were aligned using CLUSTALX (Jeanmougin et al. 1998). Disordered regions (1–7, 157–162, 171–174, and 431–439) are shown as red letters in ADPGKs from *Pyrococcus horikoshii* OT3. α -Helices (α 1– α 17, green), β -sheets (β 1– β 18, yellow), and 3_{10} helices (3_{10} 1– 3_{10} 3, red) are shown. Conserved residues among ADPGK are shown by asterisks.

Results and Discussion

Overall structure

The structure of phGK was determined by a single isomorphous replacement with an anomalous scattering (SIRAS) method and was refined at 2.0-Å resolution to an R-factor (R_{free}) of 21.9% (28.0%). The three-dimensional structure of the N-terminal region (1–6), including the His-tagged linker sequence and the three surface loops (157–162, 171–174, and 431–439), could not be modeled in this study because of poor electron density. This means that 25 residues of 457 amino acids were disordered. The present model contains the ordered residues of 7–156, 163–170, 175–430, 440–457, and 394 water molecules. The whole structure can be divided into a large α/β -domain and a small domain (Fig. 2). The large α/β -domain consisted of 11-stranded β -sheets surrounded by 13 α -helices and three 3_{10} helices ($\alpha 1$ – $\beta 1$: $\alpha 6$ – $\beta 5$ – $\alpha 7$ – $\beta 6$ – $\beta 7$ – $3_{10} 1$: $\alpha 8$ – $\alpha 9$ – $\beta 12$ – $3_{10} 2$ – $\alpha 10$ – $\beta 13$ – $\alpha 11$ – $\beta 14$ – $\alpha 12$ – $\alpha 13$ – $\alpha 14$ – $\beta 15$ – $\beta 16$ – $\alpha 15$ – $3_{10} 3$ –

$\alpha 16$ – $\beta 17$ – $\beta 18$ – $\alpha 17$). The small domain consisted of seven-stranded β -sheets and four α -helices ($\beta 2$ – $\alpha 2$ – $\alpha 3$ – $\alpha 4$ – $\beta 3$ – $\alpha 5$ – $\beta 4$: $\beta 8$ – $\beta 9$ – $\beta 10$ – $\beta 11$).

Comparisons between the structure of apo-phGK and ADP-tlGK

The structure of tlGK with ADP was recently reported (Ito et al. 2001). The structure was compared with the apo-phGK structure (Fig. 3A). The overall structure of the two enzymes was similar to that expected from the amino acid sequence. The internal structure of the domains was essentially the same (root mean square deviation, 1.14 Å and 0.58 Å for the C α atoms of 310 residues of the large domain and 103 residues of the small domain, respectively). A marked difference, however, was observed in the relative position of each domain. When the large domain of the apo-phGK structure was superimposed on that of the ADP-tlGK structure, a large movement of the small domain could be seen, and it was >5 Å on average. The distances between identical CA atoms of apo-phGK and ADP-tlGK in relation to the residue number are plotted as shown in Figure 4. The largest separation was observed for Glu46 (9.9 Å) and Asp192 (10.2 Å). The ADP-binding site is composed of two loops, a large ADP-binding loop (Thr437-Ile453 in tlGK) and a small loop (His352-Tyr357 in tlGK). In phGK, the large ADP-binding loop (430–439) was disordered under ADP-free conditions (Fig. 3A). This indicates that the loop is closely related to ADP binding and catalytic activity. The ordered large loop contacts the small domain, and consequently, it allows a closed conformation. In the small loop, the main-chain conformation is basically the same between the two structures. However, there is a marked difference in the orientation of the Tyr354 side-chain on the small loop. Tyr354 holds one water molecule instead of ADP in apo-phGK.

There is currently no available structure for ADPGK-bound glucose. However, the superposition of ribose-RK and apo-phGK gave us useful information about the glucose-binding site, despite their different phosphorylation donors, ATP and ADP, respectively. Using this information, together with that of the structural alignment of phGK and tlGK, we could predict the glucose-binding site. The conserved residues between phGK and tlGK are Asn35, Asp37, Glu91, Gly 114, Gly115, Gln116, His179, Ile202, His238 (Gln243 in tlGK), and Asp443 (Fig. 3B). The two glycine residues (Gly 114 and Gly115), located in the center of the binding site, are the conserved motif of the RK family and are regarded as a switch of the induced fit of the enzyme (Schumacher et al. 2000). The binding site appears to be formed by these residues, and the conformational change also occurs to surround the glucose, producing the hydrophobic environment of glucose for catalysis. It is better to note that the refined structure contains a sulphate ion, which

Fig. 2. (A) The stereographic CA plot of apo-phGK (ADP-dependent glucokinase from *Pyrococcus horikoshii* OT3). Every 20 residues are labeled. (B) The stereographic ribbon plot of apo-phGK. The rainbow drawing shows N-terminal as blue and C-terminal as red. The images were prepared using Molscript (Kraulis 1991) and Raster3D (Merritt and Murphy 1994).

Fig. 3. (A) The apo-phGK (ADP-dependent glucokinase from *Pyrococcus horikoshii* OT3) structure (yellow) is stereographically superimposed on that of the holo-tlGK (ADP-dependent glucokinase from *Thermococcus litoralis*; green). The large α/β -domain of the apo-phGK structure is superimposed on that of the holo-tlGK. Three disordered loops (in red) are 157–162, 171–174, and 431–439. (B) Stereographic close-up of the active site of the apo-phGK. The large ADP-binding loop (431–439) is disordered (red). The ADP of tlGK was built-in for a better understanding. The SO_4^{2-} is shown in gold. Highly conserved residues between phGK and tlGK are shown as a ball-and-stick model and are labeled. The images were prepared using Molscript (Kraulis 1991) and Raster3D (Merritt and Murphy 1994).

is LiSO_4 , used for the crystallization, because it is within the glucose-binding site (Fig. 3B). The presence of sulphate ion was indicated by the experimental map and the Fo-Fc map contoured at four and five σ levels, respectively. On the other hand, Asp443 appears to play a critical role in the glucose binding because the same residue Asp255 in RK interacts with the 5' OH of ribose. In the case of hexokinase (Anderson et al. 1978), adenylate kinase, and phosphofructokinase (Evans and Hudson 1979), the carboxylate group of an aspartic acid residue is hydrogen-bonded to the phosphoryl acceptor and functions as a general base catalyst (Anderson et al. 1979). It appears that the Asp443 in phGK plays a crucial role in abstracting a proton from the O6'-hydroxyl group of glucose.

The similarity of the structure and the substrate-induced fit with the RK and AK

The overall structure of phGK is similar to the two ATP-dependent kinases, which are RKs (Sigrell et al. 1998) and AKs (Matthews et al. 1998; Schumacher et al. 2000), although there is no apparent sequence similarity. There is less structural similarity in the small domain compared with the large domain (Figs. 3, 5). The small domain of RK consists of β -sheets only and plays a crucial role as a hook for the dimerization of RK. In phGK, tlGK, and AK, the small domain consists of a mixed α/β -structure, so that the additional α -helices disturb the dimerization. phGK exists as a monomer based on the gel filtration results (data not

Fig. 4. Plot of distances between identical CA atoms of apo-phGK (ADP-dependent glucokinase from *Pyrococcus horikoshii* OT3) and holo-tlGK (ADP-dependent glucokinase from *Thermococcus litoralis*) relative to the residue number. Two structures were superimposed on the identical residues of the large domain (7–34, 115–175, 207–242, 249–318, 322–412, and 415–457 in phGK). Red bar indicates the small-domain region (37–112 and 178–206).

shown), and the crystallographic results show that one molecule exists in an asymmetric unit. It is interesting to note that only *P. furiosus* GK among ADPGK exists as a dimer, indicating the structural difference compared with other ADPGKs (Ito et al. 2001).

A large conformational change with a 20° rotation of the small domain was observed between the apo-phGK and ADP-tlGK (Fig. 3A). Ribose-ADP-RK, ribose-RK, and apo-RK were reported in the case of RK (Sigrell et al. 1998, 1999). On the other hand, adenosine-AMP-PCP (nonhydrolyzable ATP analog)-AK, adenosine-AK, iodotubercin-AK, and apo-AK structures were reported in the case of tgAK (Schumacher et al. 2000). A marked conformational change was observed between the apo and holo enzymes. When the apo and holo structures were superimposed on the large α/β -domain, small-domain movements were observed in both RK and AK (Fig. 5A,B). A 17° rotation of its small domain occurred at the ATP binding. A similar small-domain rotation of 30° was induced by substrate binding in tgAK. The conformational change appeared to be essential for the catalytic mechanism in ADPGK, as well as RK and AK. It is important to note that the topology of ADPGK was similar, but the structure was clearly different from those of RK and AK (Figs. 3, 5). It was reported that the structure of apo-tlGK showed no large conformational change (0.4-Å

movement of the large loop) compared with that of the holo structure (Ito et al. 2001). The apo crystal of tlGK was prepared by soaking the holo crystal in ADP-free buffer; consequently, the apo structure appears to be trapped in a conformation similar to the holo in the crystal. They concluded that the ADP-tlGK structure is an open form because (1) it is similar to the apo structure of RK, and (2) no conformational change was shown between the ADP-tlGK and apo-tlGK. They also suggested that glucose induces closing, but ADP does not. However, our present structure of the apo-phGK clearly provided an open structure in contrast to ADP-tlGK. Crystals of ADP-phGK or glucose-phGK, which is the same parameter of the apo crystal, could not be obtained by the co-crystallization and soaking trials. Thus, this indicates that not only glucose but also ADP induces a large conformational change from the apo structure in ADPGK.

Conclusions

The large substrate-induced conformational change in kinase was initially introduced for yeast hexokinase, adenylate kinase, and phosphoglycerate kinase (Anderson et al. 1979). Anderson et al. proposed that the change may provide a mechanism by which some of these enzymes reduce

Fig. 5. (A) Stereographic superimposing of the apo (yellow, 1DH2) and holo (green, 1DGY) of *Toxoplasma gondii* adenosine kinase. The large α/β domain of the apo *T. gondii* adenosine kinase is superimposed on that of the holo form with adenosine and phosphomethylphosphonic acid adenylate ester. (B) Similar stereographic superimposition between the apo *Escherichia coli* ribokinase (yellow, 1RKA) and the holo form with ribose and ADP (green, 1RKD). The directions of both images are the same as those of ADP-dependent glucokinase in Figs. 2 and 3. The images were prepared using Molscript (Kraulis 1991) and Raster3D (Merritt and Murphy 1994).

their inherent adenosine triphosphatase activity and could be a general mechanism of the kinase action. Recently, it was shown that a similar mechanism exists in the ribokinase family, such as RK and AK (Sigrell et al. 1998). A proposed ordered sequential mechanism in RK is as follows (Sigrell et al. 1998): Sugar binds initially onto the large domain at an open active site, displacing water and encouraging a more closed position of the lid. Smaller conformational changes were observed to affect the nearby nucleotide-binding site, shifting it toward the structure seen when the nucleotide is bound. Once the ternary complex is formed, another rearrangement could bring the substrates close enough for nucleophilic attack of the ribose O5' on the γ -phosphorus of ATP. It was suggested that a similar and large conformational change exists for ADPGK. Not only glucose but also ADP stabilizes the loop and induces the conformational change. The large ADP-binding loop was flexible and disordered in the apo state. It appears to create a crucial environment for the kinase reaction. In the reaction mechanism,

it is not adequate to consider the same scheme, which is an ordered sequential mechanism, proposed in RK, because ADP could bind initially without glucose in ADPGK. However, it is easy to expect the cooperative-induced fit accompanying the ADP and glucose binding. The precise reaction mechanism will be solved by the structures of glucose-ADPGK and glucose-ADP-ADPGK taken together in the analysis of the enzymatic reaction.

Materials and methods

Gene cloning, purification, crystallization, and data collection

The gene (PH0589) encoding the ADPGK homolog, which shows high similarity to that of the *P. furiosus* ADPGK, was identified in the *P. horikoshii* genome (Kawarabayashi et al. 1998). The plasmid DNA pTrcHis, which carries an N-terminal His-Tag sequence, was obtained from Invitrogen. The following set of oligonucleotide primers was used to amplify the ADPGK gene fragment by PCR: The primer (5'-CCGCTGCAGATGACGAATTGGGAAAGTC TATAT-3') introduced a unique *Pst*I restriction site; the other (5'-GGCAAGCTTTTCAGTGAAGAGAAAACCTCGGAAAC-3'), a unique *Hind*III restriction site. The chromosomal *P. horikoshii* DNA was isolated as described previously (Sambrook et al. 1989) and used as the template. The amplified 1.4-kb fragment was digested with *Pst*I and *Hind*III and ligated with the expression vector pTrcHis linearized with *Pst*I and *Hind*III to generate pHGK. The *E. coli* strain JM109 (Stratagene) was transformed with pHGK. The transformants were cultivated at 37°C in 2 L Luria Bertani medium containing 50 μ g/mL ampicillin until the optical density at 600 nm reached 0.6. Isopropyl- β -D-thiogalactopyranoside was added to induce the enzyme to the medium, and the cell cultivation was continued for 3 h. Cells were harvested by centrifugation, suspended in 10 mM Tris/HCl buffer (pH 8.0), and disrupted by ultrasonication. The crude extract was heated at 80°C for 30 min, and the denatured protein was then removed by centrifugation (15,000g for 15 min). The supernatant solution was dialyzed against 20 mM Tris/HCl buffer (pH 7.5) containing 0.5 M NaCl and 10 mM imidazole, and loaded on a Ni-charged chelating-Sepharose column (2.6 $\times$ 10 cm; Pharmacia) equilibrated with the same buffer. Protein was eluted with a 200-mL linear gradient of 0 to 0.5 M imidazole in the same buffer. The active fractions were pooled, dialyzed against 10 mM Tris/HCl buffer (pH 8.0), and used as the purified enzyme preparation. ADPGK and ADPPFK activities were assayed at 50°C, essentially as described by Kengen et al. (1994). ADPGK was determined by measuring the formation of NADPH by the coupling assay using glucose-6-phosphate dehydrogenase from yeast. The assay mixture contained 100 mM Tris/HCl buffer (pH 7.5), 2 mM $MgCl_2$, 1 mM NADP, 20 mM glucose, 2 mM ADP, 1 unit of glucose-6-phosphate dehydrogenase, and the enzyme. The absorbance of NADPH was followed at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

All crystallization experiments were performed at 20°C using the hanging drop vapor diffusion method, in which 3 μ L of 10 mg/mL protein solution was mixed with an equal volume of mother liquor, which included ~9% to 13% polyethylene glycol 6000, 0.2 M $LiSO_4$, and 0.1 M citrate buffer (pH 3.6). The crystal belonged to the orthorhombic space group *P*212121 with following unit cell parameters: a, 64.7 Å; b, 74.7 Å; and c, 99.2 Å. Heavy atom derivatives were prepared by soaking the crystals in a reser-

voir solution containing HgCl_2 . Data were collected using an ADSC Quantum4R CCD detector system (Area Detector Systems) on the BL-6A beamline at the Photon Factory in Tsukuba, Japan (Table 1), at room temperature using a glass quartz capillary. Data processing was performed using DPS/mosflm (Rossmann and van Beek 1999) and scala (CCP4 1994).

Phasing and refinement

Phase calculations were performed with SOLVE (Terwilliger and Berendzen 1999) using one mercury derivative with anomalous data at 2.0-Å resolution. The SIRAS map at 2.0 Å (figure of merit [FOM] 0.40; score, 8.6) was further improved by maximum-likelihood density modification using RESOLVE (Terwilliger 2000). The resulting electron density map was of excellent quality and was subjected to the ARP/wARP for auto-tracing. A thousand cycles of warpNtrace produced the main-chain tracing of 310 amino acids with a connectivity index of 0.85, and the successive side-chain tracing produced 172 amino acids with an average confidence level of 0.12. This model was not perfect; however, it was helpful for the next manual tracing using program O (Jones et al. 1991). After the iterative cycles of the manual rebuilding and the refinement using CNS (Brunger et al. 1998), the final R_{cryst} and R_{free} were 21.9% and 28.0%, respectively (Brunger 1992). PRO-CHECK (Laskowski et al. 1993) produced results with 91.3% of the residues in the most favored region and only one residue: Tyr32 in the disallowed region. Tyr 32 was located in the core consisting of Val214, His255, and Val252. Tyr37 in tLGK was an identical residue and showed a similar ϕ/ψ angle. The final model had 430 residues with 394 water molecules. Residues in the N-

terminal (1–6) and three flexible surface loops (157–162, 171–174, and 431–439) were not modeled into the current structure.

Coordinates

The coordinates have been deposited in the RCSB Protein Data Bank (accession code 1L2L).

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Table 1. Statistics on data collection, phase determination and refinement

Data collection	Native	Hg
Max resolution (Å)	2	2
UniqueReflections	152678	275056
Redundancy	4.7	8.3
Completeness (%)	97.5	99.1
R symm (%)	0.057	0.09
I/σ (I)	9.8	5.4
Last shell		
Resolution range (Å)	2.11–2.00	2.11–2.00
Completeness (%)	90.4	97.1
R symm (%)	0.207	0.418
SIRAS Phasing		
FOM	0.40	(20–2.0 Å)
DM-FOM	0.74	(20–2.0 Å)
Refinement		
Resolution (Å)	15–2.0	
R factor (%)		
R _{cryst} (%)		0.219
R _{free} (%)		0.28
No. of protein atoms		3482
No. of water molecules		394
Rmsd in bond lengths (Å)		0.006
Rmsd in bond angles (°)		1.2
Average B factor (Å ²)		32.6

Crystal is $P 212121$ $a = 64.7$ Å, $b = 74.7$ Å, $c = 99.2$ Å, $\alpha = \beta = \gamma = 90^\circ$. Data were collected at Photon Factory BL6A using $\lambda = 1.00$ Å; R symm = $\sum h \sum i |I_h - \langle I_h \rangle| / \sum I_h$.

- nase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Biol. Chem.* **270**: 30453–30457.
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