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Glutamate 83 Is Important for Stabilization of Domain–Domain Conformation of *Thermus aquaticus* Glycerol Kinase

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The gene *glpK*, encoding glycerol kinase (GlpK) of *Thermus aquaticus*, has recently been identified. The protein encoded by *glpK* was found to have an unusually high identity of 81% with the sequence of GlpK from *Bacillus subtilis*. Three residues (Arg-82, Glu-83, and Asp-244) of *T. aquaticus* GlpK are conserved in all the known GlpK sequences, including those from various bacteria, yeast and human. The roles that these three residues play in the catalytic mechanism were investigated by using site-directed mutagenesis to produce three mutants: Arg-82-Ala, Glu-83-Ala, and Asp-244-Ala. Replacement of Asp-244 by Ala resulted in a complete loss of activity, thus suggesting that Asp-244 is important for catalysis. Taking k_{cat}/K_m as a simple measure of catalytic efficiency, the mutants Arg-82-Ala and Glu-83-Ala were judged to cause 190- and 37,000-fold decrease, respectively, when compared to the wild-type GlpK. Thus, these three residues play a critical role in the catalytic mechanism. However, only mutant Glu-83-Ala was cleaved by α-chymotrypsin, and proteolysis studies showed that the mutant Glu-83-Ala involves a change in the exposure of Tyr-331 at the α-chymotrypsin site. This indicates a large domain conformational change, since the residues corresponding to Glu-83 and Tyr-331 in the *Escherichia coli* GlpK sequence are located in domain IB and domain IIB, respectively. The apparent conformational change caused by replacement of Glu-83 leads us to propose that Glu-83 is an important residue for stabilization of domain conformation.

Key words: α-chymotrypsin, domain conformational change, glycerol kinase, site-directed mutagenesis, *Thermus aquaticus*.

Glycerol kinase (GlpK) from *Escherichia coli* catalyzes the MgATP-dependent phosphorylation of glycerol to produce *sn*-glycerol-3-phosphate and is the rate-limiting enzyme in the utilization of glycerol (*1*, *2*). Its catalytic activity is inhibited by the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) phosphocarrier protein IIA^{Glc} (*3*, *4*) and the glycolytic intermediate fructose 1,6-bisphosphate (*5*). Its activity is also regulated by the membrane-bound glycerol facilitator (GlpF) protein (*6*). Recently, we have cloned and sequenced the *glpK* from *Thermus flavus* and *Thermus aquaticus* (*7*, *8*) and found that *glpF* is located directly upstream of *glpK*. We also found that *T. flavus* is capable of taking up glycerol, that *glpF* and *glpK* are expressed constitutively, and that glucose exerts a repressive effect on the expression of these genes (*9*). Surprisingly, the thermostable GlpK and GlpF showed an unusually high degree of sequence identity with GlpK and GlpF of *Bacillus subtilis*, but residues 472–476 of GlpK (equivalent to 473–477 in the *E. coli* GlpK), the putative IIA^{Glc}-binding site, are not conserved. Pettigrew *et al.* have recently constructed a mutant of the *T. flavus* GlpK carrying the regulatory binding site for IIA^{Glc} from the *E. coli* GlpK. The mutant enzyme retains activity and is expressed well, but it is not inhibited by IIA^{Glc} from *E. coli* and does not bind IIA^{Glc} (D.W. Pettigrew, personal communication).

T. flavus was found to possess the general components of the phosphoenolpyruvate:sugar phosphotransferase system enzyme I and histidine-containing protein (HPr), these proteins catalyse the phosphorylation of *T. flavus* GlpK (*9*). In contrast to PEP-dependent phosphorylation of enterococcal GlpK, which stimulated GlpK activity about 10-fold (*10*), phosphorylation of *T. flavus* GlpK caused only a slight increase in enzyme activity. Since there are at present no functional data on the active sites of thermostable GlpK and how the activity is regulated is rather unclear, we therefore mutated the Arg-82, Glu-83, and Asp-244 residues, which are conserved in all the known GlpK sequences, including those from various bacteria, yeast (*11*) and human (*12*), to evaluate the role of these three residues in the catalytic mechanism.

MATERIALS AND METHODS

Recombinant DNA Methods—Molecular biology procedures were carried out as described by Sambrook *et al.* (*13*), except as noted. Restriction endonucleases and various DNA modifying enzymes were purchased from Takara Shuzo (Kyoto) and Toyobo (Osaka) and used in accordance with the manufacturers' instructions. DNA sequencing was performed by the Sanger dideoxy method (*14*) with an autocycle sequencing kit (Pharmacia). All materials and reagents used were of molecular biology grade.

Site-Directed Mutagenesis—Mutagenesis was performed according to Ito *et al.* (*15*) using the PCR *in vitro* mutagene-

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Abbreviations: PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride.

sis kit from Takara Shuzo. The *glpK* gene on an *Eco*RI–*Hin*dIII fragment from pTAGYK20 (*8*) was cloned into the same enzyme site of pBluescript SK(–) and used as the template. The oligonucleotide primers used and the respective changes (underlined) obtained were as follows: Arg-82-Ala, 5′-ACGAACCAGGCGGAGACGACGGT-3′; Glu-83-Ala, 5′-GAACCAGCGGGCGACGACGGTG-3′; Asp-244-Ala, 5′-AGCCGCGGGCGCCAGCAGGC-3′. Mutations were confirmed by DNA sequencing, and the successful mutations were cloned back into the *Eco*RI–*Hin*dIII site of pKK223-3 expression vector (*16*). *E. coli* strain XL1-Blue was used for all DNA manipulations except for expression.

Enzyme Purification—E. coli strain KH18 was constructed by placing the *glpK* deletion into the chromosome of strain MC4100 to provide a *glpK*-deletion background for expression of *T. aquaticus* GlpKs. The strain KH18 was obtained from Donald W. Pettigrew of the Department of Biochemistry and Biophysics, Texas A&M University. All the mutant *glpK* genes were overexpressed under the control of the strong *tac* promoter of plasmid pKK223-3 in KH18. However, it should be noted that the plasmid pREP4 present in KH18, which carries the *lacI* gene (*17*), ensures high levels of protein expression using pKK223-3. Mutant GlpKs were purified as previously described (*8*, *18*), with modifications. *E. coli* KH18 cells harboring a mutant *glpK* gene plasmid were grown overnight at 37°C in 200 ml of Luria-Bertani medium containing ampicillin (50 μg/ml) and streptomycin (50 μg/ml). Cells were harvested by centrifugation (5,000 ×*g*; 15 min) and sonicated (ultrasonic disruptor UD-200, Tomy) in 50 ml of extraction buffer (20 mM Tris-Cl, pH 7.5). The cell debris was eliminated by centrifugation at 28,000 ×*g* for 30 min. The supernatant was subjected to ammonium sulfate precipitation and hydrophobic chromatography on Toyopearl HW65C, followed by FPLC on Mono Q column. Wild-type GlpK was purified as previously described (*8*). The final purities of the GlpKs were checked by SDS-PAGE using Coomassie Brilliant Blue and judged to be purified to homogeneity.

Analytical Methods and Enzyme Assay—Protein concentrations were determined as described by Bradford (*19*) or by measuring A_{280}. Proteins were analyzed by denaturing (SDS) or native polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (*20*). GlpK activity was measured as described previously (*8*). One unit of GlpK activity was defined as the amount of enzyme that catalyzed production of 1 μmol of glycerol 3-phosphate per min. The K_m (Michaelis constant) and k_{cat} (catalytic constant) values were estimated from Lineweaver-Burk plots.

Proteolysis Studies—Wild-type and mutant GlpKs (2 mg/ml) were incubated with α-chymotrypsin (final concentration, 10 μg/ml; Sigma) in extraction buffer at 37°C. At the indicated times, aliquots of the reaction mixture were quenched with 1 mM PMSF (Nacalai Tesque, Kyoto). An equal volume of 1 × SDS-PAGE sample loading buffer was added, and the samples were heated at 98°C for 5 min prior to electrophoresis on 12.5% SDS-PAGE gel.

Identification of the Amino Acid Sequence Cleaved by Protease—Chymotrypsin cleavage products were separated by SDS-PAGE and transferred onto Clearblot-P membrane (Atto, Tokyo). The membrane was stained with Coomassie Brilliant Blue, visualized bands of interest were excised from the membrane with a scissors, and sequences were determined by using an Applied Biosystems model 492 protein sequencer.

TABLE I. **Kinetic parameters of wild-type and mutant enzymes.**[a]

Enzyme	K_m (M)	k_{cat} (s^{-1})[b]	k_{cat}/K_m (s^{-1} M^{-1})
Wild type	9.1×10^{-5}	1.5×10^{2}	1.7×10^{6}
Arg-82-Ala	8.3×10^{-5}	7.5×10^{-1}	9.0×10^{3}
Glu-83-Ala	5.0×10^{-3}	2.3×10^{-1}	4.6×10^{1}
Asp-244-Ala	NA[c]	NA	—

[a]Kinetic values were determined using glycerol as substrate. [b]k_{cat} was calculated assuming a molecular mass of 109.6 kDa. [c]NA, no detectable activity.

RESULTS

Kinetic Study of the Wild-Type and Mutant GlpKs—To assess their role in catalysis and glycerol binding, Arg-82, Glu-83, and Asp-244 were each mutated to alanine, which cannot participate in hydrogen bonding with the hydroxyl groups of glycerol. The kinetic parameters of the wild-type and three mutant enzymes are summarized in Table I. The replacement of Asp-244 by Ala abolished activity completely, consistent with the fact that Asp-245 of *E. coli* GlpK acts as a general base for the catalysis (*21*, *22*). It has been suggested that the role of this aspartate is consistent with that of Asp-209 and Asp-657 in the N- and C-terminal halves of mammalian hexokinase II (*23*), Asp-657 in the C-terminal half of tumor hexokinase (*24*), Asp-205 in hepatic glucokinase (*25*), and Asp-211 in yeast hexokinase (*26*, *27*).

Arg-83 and Glu-84 in the *E. coli* GlpK (equivalent to Arg-82 and Glu-83 in the *T. aquaticus* GlpK) form hydrogen bonds with glycerol (*21*). Taking k_{cat}/K_m as a simple measure of catalytic efficiency, mutants Arg-82-Ala and Glu-83-Ala were judged to cause 190-fold and 37,000-fold decrease when compared to the wild-type GlpK. The replacement of Glu-83 by Ala resulted in a 652-fold decrease in k_{cat} and a 55-fold increase in K_m, suggesting that Glu-83 is important for both catalysis and substrate binding. On the other hand, the replacement of Arg-82, immediately adjacent to Glu-83, by Ala decreased k_{cat} 200-fold without affecting the K_m. This decrease in the k_{cat} values for mutants Arg-82-Ala

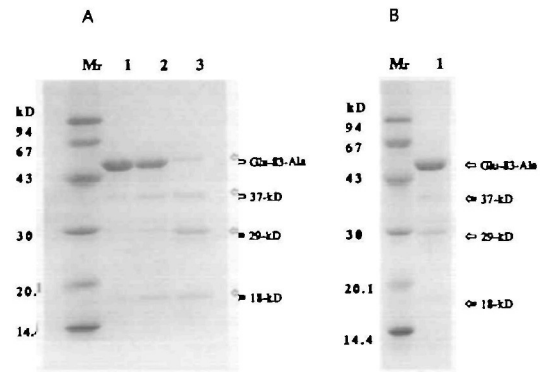

Fig. 1. **Proteolysis of Glu-83-Ala by α-chymotrypsin.** A: Glu-83-Ala was digested with α-chymotrypsin under the conditions described under "MATERIALS AND METHODS" at 37°C for 1, 5, and 60 min (lanes 1–3). B: Glu-83-Ala was digested with α-chymotrypsin at 37°C for 60 min in the presence of 2 mM ATP. The positions of mutant Glu-83-Ala, 37-kDa, 29-kDa, and 18-kDa fragments are shown, along with molecular mass standards in the lane M_r.

and Glu-83-Ala by two orders of magnitude might be due to loss of the hydrogen bonds with glycerol, which contribute to maintaining glycerol in the correct position and so facilitate nucleophilic attack on the γ-phosphate of ATP.

Altered Properties of the Mutant—We have reported that the GlpK from *T. aquaticus* and *T. flavus* possessed a high thermal stability (*7*, *8*, *18*). The activity of wild-type GlpK from *T. aquaticus* was not changed by incubation at 60°C for 15 min, but the stability of the mutant Glu-83-Ala was greatly reduced and 10 degree lower than that of wild-type. It is likely that a conformational change has occurred in this mutant. In addition, we have previously shown that the GlpK from *T. flavus* was inhibited by PCMB (*18*). *T. aquaticus* GlpK was also inhibited by 4 μM PCMB. However, the mutant Glu-83-Ala was not inhibited by this concentration of PCMB. The change in susceptibility to this bulky reagent could be the result conformational change caused by the mutation. Despite this insight, little is known about the mechanism of the inhibitory action of PCMB in GlpK.

Apparent Conformation of Mutated-GlpK—In the related proteins Dnak (*28*) and *E. coli* GlpK (*29*), proteolysis has been used to examine the conformational change due to ATP binding. We also used this method to examine the conformational changes occurring in mutant GlpKs. Treatment of Glu-83-Ala with α-chymotrypsin caused cleavage of the M_r 54.8 kDa enzyme into three major bands having molecular masses, estimated by SDS-PAGE, of 37, 29, and 18 kDa (Fig. 1A). During the digestion, the 37- and 18-kDa bands appeared first, and the 29-kDa band appeared later. In the presence of 2 mM ATP, Glu-83-Ala was cleaved much more slowly, and a substantial amount of the intact 54.8-kDa band was detected even after 60 min of incubation (Fig. 1B). In contrast, no cleavage occurred during the incubation of wild-type, Arg-82-Ala, and Asp-244-Ala with α-chymotrypsin under the same conditions (data not shown). Furthermore, the electrophoretic mobilities of the Glu-83-Ala and Asp-244-Ala in native PAGE were significantly slower than those of wild-type and Arg-82-Ala GlpKs (Fig. 2).

Determination of the Cleavage Sites of Glu-83-Ala—The 37-kDa, 29-kDa, and 18-kDa bands of chymotrypsin cleavage product were sequenced to yield MNQYILAIDQ, MNQYILAIDQ, and AEKVESTDGV, respectively. The 37- and 29-kDa bands both start with the initiating methionine (*8*). The first 10 amino acids of the 18-kDa band correspond to nucleotides at positions 332–341 in the *T. aquaticus* GlpK. These results immediately suggested the existence of two α-chymotrypsin cleavage sites within the Glu-83-Ala, with cleavage at one site generating the 18-kDa fragment plus the 37-kDa intermediate, which was further cleaved at an another site to yield the 29- and 8-kDa fragments (the smallest band is not visible in the figure). Moreover, immediately adjacent to the N terminus of the 18-kDa fragment is the Tyr-331 residue, as is to be expected considering the specificity of this protease.

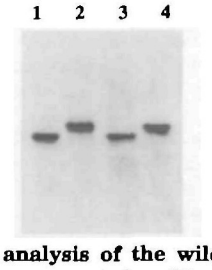

Fig. 2. **Native PAGE analysis of the wild-type and mutant GlpKs.** The proteins were separated on 7% native polyacrylamide gels at 50 mA and room temperature. Samples of 5 μg of proteins were used. The gel was stained with Coomassie Brilliant Blue. Lanes 1 to 4, Arg-82-Ala, Glu-83-Ala, wild-type enzyme, and Asp-244-Ala, respectively.

DISCUSSION

We constructed three site-directed mutants of *T. aquaticus* GlpK. Asp-244 appeared to be essential for catalysis as expected from the sequence alignments and confirmed in this report that the replacement of a single aspartate residue abolished the enzyme activity completely. Two aspartate residues (Asp-245 and Asp-10 in *E. coli* GlpK; Asp-244 and Asp-9 in *T. aquaticus* GlpK) in the immediate vicinity of the Mg^{2+} ion are conserved throughout the superfamily of sugar kinases, Hsc70, and actin (*21*, *22*). The effect of mutant Asp-244-Ala is unlikely to be due to change in Mg^{2+} binding, because the conserved residue which interacts with the Mg^{2+} ion of the bound Mg^{2+}-nucleotide complex is aspartate 9 in *T. aquaticus* GlpK. Asp-244 probably acts to remove a proton from the 3-hydroxyl group of glycerol and orientates the nucleophilic attack on the γ-phosphate of ATP molecule correctly, because mutant Asp-244-Ala experienced a conformational change as showed by the fact that the electrophoretic mobility of this mutant in native PAGE was significantly slower than that of the wild-type. Since α-chymotrypsin did not cleave the Asp-244-Ala, this conformational change is different from that of Glu-83-Ala.

Proteolysis studies showed that the Glu-83-Ala mutation involves a conformational change that leads to exposure of Tyr-331 upon α-chymotrypsin cleavage. The corresponding residue in the *E. coli* GlpK sequence is also Tyr-331, which lies in domain IIB (*21*, *30*), while Glu-84 in the *E. coli* GlpK, equivalent to Glu-83 in the *T. aquaticus* enzyme, is located in domain IB. Significantly, it has been suggested that changing Glu-83 to Ala in the *T. aquaticus* GlpK caused a large domain conformational change. This is also consistent with the finding that Glu-83-Ala migrated slowly in native PAGE (Fig. 2), since external localization of the Tyr-331 region would alter the surface charge.

Glycerol kinase belongs to the superfamily of sugar kinases, Hsc70, and actin. In the superfamily of Hsc70 and actin, it has been predicted that a number of small residues serve as a "hinge" about which the domains rotate (*31*). The possibility that the mutated residue Ala-83 acts as a hinge residue can be excluded, because Glu-84 in the *E. coli* GlpK, equivalent to Glu-83 in the *T. aquaticus* GlpK, is not located at a key contact point between the two α-helices at the domain interface mentioned in Ref. *31*. We consider it likely that Glu-83 forms hydrogen bonds with both the hydroxyl group of glycerol and the side chain of Arg-187 (equivalent to Arg-188 in the *E. coli* GlpK) (Fig. 3A). The second side chain of Arg-187 might form a hydrogen bond with Glu-302 (equivalent to Glu-303 in the *E. coli* GlpK), which lies in the β-α-α loop (Fig. 3B, the loop is indicated in dark). The interaction of the two domains is proposed to involve the amino acids Glu-83, Arg-187, and Glu-302, which are conserved in all the known GlpK sequences,

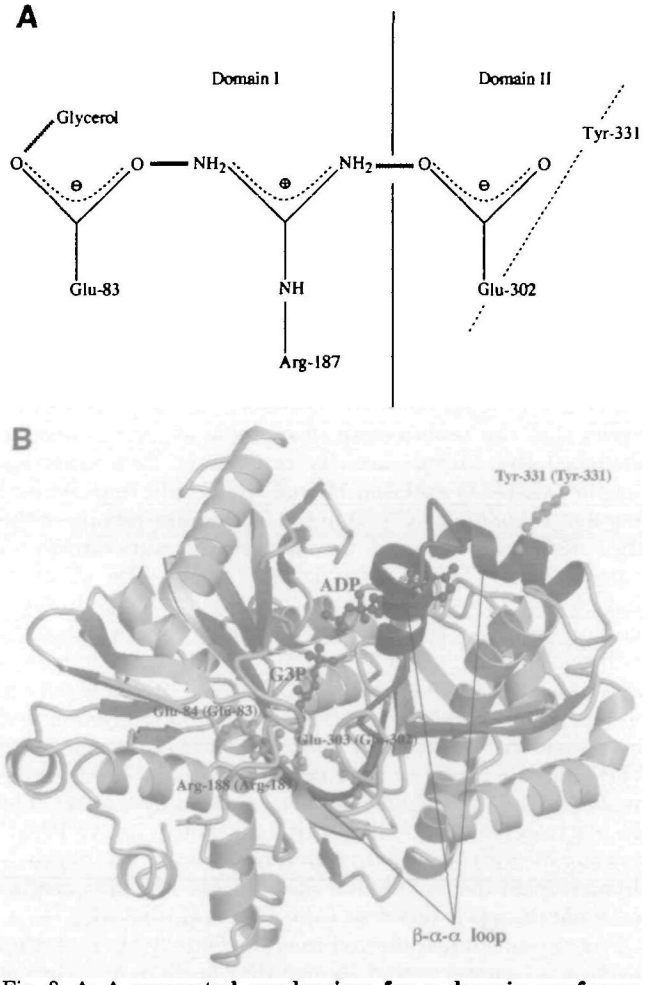

Fig. 3. **A: A suggested mechanism for a domain conformational change of mutant Glu-83-Ala. B: Three-dimensional models showing the β-α-α loop of GlpK.** This image was generated from the X-ray crystallographic coordinates (PDB 1GLA) of Hurley *et al.* (*21*). The corresponding residues in the *T. aquaticus* GlpK are given in parentheses. Figure generated using MOLSCRIPT.

including those from bacteria, yeast, and human. The conformational change appears to be best described as a movement of the β-α-α loop containing Tyr-331 from the rigid body model: the exposure of Tyr-331, making is accessible for α-chymotrypsin cleavage, suggests that the substitution of Ala for Glu-83 abolished the link between Glu-83 and the β-α-α loop *via* Arg-187. The loss of hydrogen bonds with Glu-83 and Arg-187 would be accompanied by a slight adjustment in position of this arginine in the GlpK structure, and therefore alter the hydrogen bond with Glu-302, a key residue for movement of the β-α-α loop. We suggest that it would be especially important for Glu-83, which interacts with Arg-187 and affects Glu-302.

Pettigrew *et al.* have reported that a single amino acid mutation of Gly-304 to Ser in *E. coli* GlpK changed the conformation, catalytic, and regulatory properties, which are consistent with a role for the domain closure motion (*29*). Gly-304 is located in the β-α-α loop. The corresponding residue in *T. aquaticus* GlpK is Gly-303, immediately adjacent to Glu-302. It seems likely that the effect of this mutant is due to a movement of the β-α-α loop, which affects the interaction between the enzyme's two domains.

Arg-82 is immediately adjacent to Glu-83, but the Arg-82-Ala mutant does not show the apparent conformational change estimated by K_m, native PAGE, and proteolysis study, because the side chain of Arg-82 does not form a hydrogen bond with Arg-187, as described above. These results strongly support the idea that the β-α-α loop movement is essential for conformational change.

The superfamily of sugar kinases, Hsc70, and actin is defined by a structure possessing a high degree of similarity in the conserved ATPase core subdomains (*32*). Known crystal structures of this superfamily include those of *E. coli* GlpK (*21*), yeast hexokinase (*26*, *33*), actin (*34*), Hsc70 (*35*), and Dnak (*36*). These proteins all bind ATP and are thought to undergo a large conformational change during their function cycle. A common mechanism for the nucleotide-dependent conformational change has been described by Holmes *et al.* (*31*). Of the *T. aquaticus* GlpKs, Glu-83-Ala is cleaved more slowly in the presence of ATP (Fig. 1B), which suggests that the Glu-83-Ala undergoes a conformational change upon binding of ATP. This is reverse evidence in support of our proposition that ATP links to the β-α-α loop to prevent domain movement, since Gly-310 and Gln-314 in the *E. coli* GlpK (equivalent to Gly-309 and Gln-313 in the *T. aquaticus* GlpK), which participate in ATP binding, are located in this β-α-α loop. These facts lead us to propose that Glu-83 is an important residue not only in the substrate binding but also in stabilizing the domain–domain conformation. Other residues, such as Arg-187 and Glu-302, may be important for stabilization of domain conformations. Further mutations will have to be constructed to investigate this idea.

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