Functions of the conserved anionic amino acids and those interacting with the substrate phosphate group of phosphoglucose isomerase

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Abstract Phosphoglucose isomerase catalyzes the isomerization between glucose 6-phosphate and fructose 6-phosphate in cytoplasm, and functions as autocrine motility factor and neuroleukin outside the cells. A phosphoglucose isomerase from *Bacillus stearothermophilus* (pgiA) was subjected to mutagenesis study to address the catalytic function of the conserved anionic residues and those probably interacting with the phosphate group of substrates. The results suggest that Glu290 works concertedly with His311 as a general acid–base pair to initiate the isomerization step, and Glu150 assists the base function of His311. The conserved loop structure consisting of Gly205–Gly206–Arg207 plays a critical role for the recognition of substrates.

Key words: Phosphoglucose isomerase; Glucose 6-phosphate isomerase; Autocrine motility factor; Aldose–ketose isomerization; *Bacillus stearothermophilus*

1. Introduction

Phosphoglucose isomerase (EC 5.3.1.9) is a protein with multiple functions. As being a glycolytic enzyme, it catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate in the glycolysis and gluconeogenesis pathways. The deficiency of this metabolic enzyme affects the metabolism of red blood cells and consequently leads to non-spherocytic hemolytic anemia [1]. Several mutations associated with this hereditary disease have been identified [2–4]. Outside the cells, it also functions as a neuroleukin [5,6], autocrine motility factor (AMF) of tumor cells [7,8], and the differentiation and maturation mediator (DMM) for human myeloid leukemia cells [9]. Given the involvement of phosphoglucose isomerase in so many different biological processes, its structure–function relationship is an important issue.

To achieve the aim, two isozymes of the enzyme from *Bacillus stearothermophilus* (pgiA and pgiB) have been studied in series experiments. PgiA and pgiB share 70% identity in amino acid sequence and have similar properties in terms of enzymatic activity and thermostability. Both pgiA and pgiB were purified and crystallized [10]; furthermore, the 3-D structures of pgiB [11] and its complex with 5-phosphoarabinonate [12] have been determined. Since 5-phosphoarabinonate behaved as a transition-state inhibitor of the enzyme [13], the active site of the complex probably resumes a transition-state-like configuration. In spite of the bacteria origin, the phosphoglucose isomerase from *B. stearothermophilus* was capable of functioning as neuroleukin and AMF in vitro [11]. According to the crystal structure, pgiB is in a dimeric form. Each subunit is composed of a large and a small globular domain with similar structure in which a β-sheet core is surrounded by α-helices. The active site is located within the two globular domains and the interface between the two subunits. Recently, the structure of phosphoglucose isomerase from rabbit muscle was also solved [14]. The overall fold of the rabbit muscle phosphoglucose isomerase is very similar to that of pgiB. Superposition of the two structures reveals no significant amino acid shifts at and around the active site and no significant changes in interdomain or intersubunit interfaces. Nonetheless, the inhibitors, 5-phosphoarabinonate for pgiB and 6-phosphogluconate for the rabbit enzyme, bound in different dispositions in the active site. The phosphate group of the former lies in the bottom of the active site cleft, while that of the latter occupies the entrance of the active site.

According to numerous kinetic studies [15–17], the catalytic mechanism of phosphoglucose isomerase has long been proposed to include steps of (a) binding of cyclic form of substrates, (b) ring opening of substrates, (c) base-catalyzed isomerization via a *cis*-enediol intermediate which is the rate-limiting step, (d) closure of products, and (e) release of products. The temperature dependence of pK_a values, determined from pH profiles of enzymatic activity, suggested critical roles of a protonated lysine and a non-protonated imidazole in the catalysis [18]. Mutagenesis has been carried out in pgiA to address the function of several conserved amino acids. Substitution of alanine for Arg207 and Lys425 (Arg202 and Lys420, respectively, in pgiB; hereafter, residues in parentheses indicated the corresponding position in pgiB) resulted in a 10^5- and 400-fold decrease, respectively, in the catalytic efficiency (k_{cat}/K_M) of the enzyme [19]. Mutational data also suggested the critical role of His311 (His306) [20]. The pK_a value of His311, calculated from the titration of the inactivation rate constant of pgiA by *N*-bromoacetyl ethanolamine, supports the base function of His311. In this study, we continue to investigate the contributions of the other active-site residues to substrate affinity and to enzymatic activity by site-directed mutagenesis. Focus was on the conserved anionic amino acids and those probably interacting with the phosphate group of substrates. With the guidance of the active-site architecture, the possible roles of the investigated residues in the catalytic mechanism of phosphoglucose isomerase are discussed.

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2. Materials and methods

2.1. Site-directed mutagenesis

Mutation was generated on plasmid pFDI132, which contains pgiA gene, according to a PCR-based method using a pair of 5′-phosphorylated divergent primers as described previously [19]. The mutagenic nucleotide sequence was incorporated at the 5′ end of one of the divergent primers. After PCR amplification with *Pfu* DNA polymerase, the blunt-ended linear DNA product was purified, self-ligated, and transformed back into *Escherichia coli* DF2145 [(*eda-zwf*)*15, hisG1*(*Fs*), *pgi-7*::*Mu-1, rpsL115, hsdR2, zjj202*::Tn*10*]. The desired mutation was confirmed by determining the nucleotide sequence with Amplicycles sequencing kit (Perkin-Elmer).

2.2. Production and purification of enzymes

E. coli strain DF2145, which has a disruption in its own *pgi* gene, was used to produce pgiA and the variants. The culture conditions of *E. coli* cells and the purification procedure for the recombinant phosphoglucose isomerase were as described previously [10]. All purified pgiAs appeared homogeneously on SDS–PAGE. Similar elution behavior of the mutants to wild-type enzyme in each chromatographic step suggests that each single amino acid substitution did not cause global structural changes. Therefore, the change of kinetic constants upon mutation is probably resulting from the local effects, and may be used to address the function of the targeted amino acid. Protein concentration was determined by the Coomassie blue method using the determination kit obtained from Pierce (Rockford, IL, USA).

2.3. Enzyme activity determination

The activity catalyzing the isomerization of fructose 6-phosphate to glucose 6-phosphate was determined by the coupled glucose 6-phosphate dehydrogenase method [21]. The standard activity assay buffer (1 ml) contained 20 mM potassium phosphate (pH 7.0), 2 mM NAD^+, 10 units of glucose 6-phosphate dehydrogenase, and varied amount of fructose 6-phosphate and pgiA. For each reaction condition, the assay was repeated at least twice by varying the amount of pgiA to assure that pgiA is always the limiting factor for the acceleration rate of NADH. For determining the activity pH profile, imidazole buffer was used from pH 6 to 7.5, Tricine buffer from 8.0 to 8.5, and glycine buffer for pH 9.0. The steady-state kinetic constants, K_M and V_{max}, were determined from the Lineweaver–Burk and the Eadie–Hofstee plots. The catalytic constant, k_{cat}, was calculated from the equation $V_{max} = k_{cat} \times [E]_0$, where $[E]_0$ is the molar concentration of the monomer of phosphoglucose isomerase.

3. Results and discussion

3.1. Mutation of the conserved anionic residues

Genes encoding phosphoglucose isomerase have been cloned from a variety of organisms ranging from bacteria, plants to mammals. Comparison of their deduced amino acid sequences reveals several homologous regions including those documented as signature patterns ([LIVM]-G-G-R-[FY]-S-[LIVM]-X-[STA]-[STA]-[LIVM]-G and [FY]-D-Q-X-G-V-E-X-X-K) by Swiss-Prot (http://expasy.hcuge.ch/sprot/prosite.html). Mutational analysis and affinity labeling of pgiA have suggested the important roles and the active-site location of the conserved lysine, arginine, and histidine (Lys144, Lys294, Lys425, Arg207, and His311 of pgiA) [19,20]. The structures of pgiB and the rabbit enzyme confirm that all homologous regions are indeed active-site constituents. To explore the importance of other conserved residues, the anionic residues including Glu150, Glu290, Glu401, Asp417, and Glu422 in pgiA were first subjected to site-directed mutagenesis in the present study. Since $>90\%$ amino acids around the active site are identical between pgiA and pgiB, the active-site architectures of pgiB (Fig. 1) should be applicable to that of pgiA. According to this assumption, Glu150 (Glu145) is located behind His311 (His306) with one of its carboxylate oxygen atoms hydrogen bonding to $N^{\varepsilon 2}$ of His311 (His306), whose $N^{\delta 1}$ in turn is in contact with C1 carboxylate oxygen and C2 hydroxyl oxygen of 5-phosphoarabinonate. The carboxylate of Glu290 (Glu285) contacts another C1 carboxylate oxygen and the phosphate group of the inhibitor. Glu401 (Glu396) is $\sim$10 Å away from 5-phosphoarabinonate and doses not contribute itself directly to the formation of the active-site surface. The side chain of Asp417 (Asp412) points outward from the active-site cavity; however, its backbone oxygen is involved in the active-site hydrogen bonding network by forming a hydrogen bond to the guanido nitrogen of Arg207 (Arg202). One of the carboxylate oxygen atoms of Glu422 (Glu417) hydrogen bonds to the amino group of Lys425 (Lys420) which, in turn forms a hydrogen bond to O_2 of the inhibitor.

Each targeted residue was initially substituted with alanine, and the resulting changes of the kinetic constants are shown in Table 1. Substitution of alanine for Glu150 resulted in an insignificant change of K_M and a 10^3-fold decrease in k_{cat}, suggesting that Glu150 plays a critical role in catalysis although it is not directly in contact with substrates. In response to mutation of Glu290, the value of K_M increased $\sim$3-

Table 1
Kinetic constants of wild-type and mutant phosphoglucose isomerase

Enzyme	$K_M^{\text{fructose 6-P}}$ (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	$\Delta\Delta G^{\ddagger}$[a] (kcal/mol)
Wild-type	0.26 (0.05)[b]	150 (10)	580	
E150A	0.27 (0.01)	0.12 (0.02)	0.44	4.3
E150D	0.25 (0.04)	54 (10)	216	
E150N	0.49 (0.01)	0.024 (0.001)	0.05	
E150Q[c]	1	4.5×10^{-3}	4.5×10^{-3}	
E290A	0.85 (0.03)	0.36 (0.02)	0.42	4.4
E290D	0.46 (0.03)	0.036 (0.003)	0.08	
E290N	1.9 (0.2)	0.010 (0.001)	0.005	
E290Q	1.6 (0.1)	0.46 (0.02)	0.28	
E401A	0.16 (0.10)	88 (4)	550	0.03
D417A	0.25 (0.04)	16 (1)	64	1.3
E422A	0.37 (0.01)	45 (9)	120	0.95
G205L	0.42 (0.05)	71 (6)	169	
G206L[c]	10	4.3×10^{-3}	4.3×10^{-4}	
Q418A	1.6 (0.1)	7.5	4.7	2.9

[a] $\Delta\Delta G^{\ddagger}$ was calculated only for mutant enzymes that had alanine substitution for targeted amino acids according to equation $\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wt}]$.

[b] Figures in parentheses are the standard deviations.

[c] The data are the average of only two independent experiments, due to the large amount of enzyme needed for a kinetic assay.

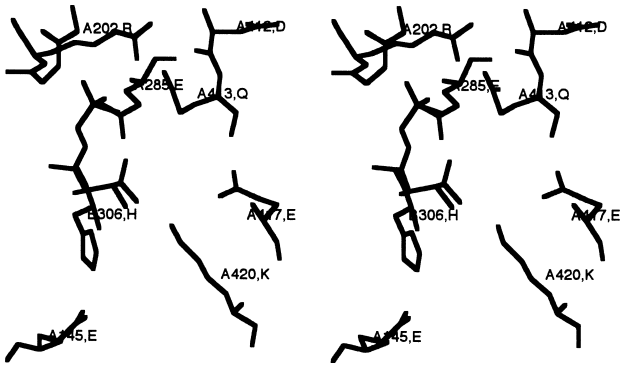

Fig. 1. Simplified drawing of the active-site structure of pgiB complexed with 5-phosphoarabinonate. The active site is composed of amino acids from two neighboring subunits; for example, His306 is from B subunit (labeled as B306, H), while other residues are from the A subunit.

fold and the catalytic efficiency (k_{cat}/K_M) dropped ~3 orders of magnitude, suggesting that Glu290 plays a role primary in stabilizing the transition-state substrate. The mutation of Glu401 to alanine had an insignificant effect on k_{cat}/K_M but a slight benefit for the binding affinity to the ground-state substrate. Mutation of Asp417 caused an insignificant change of K_M that could be attributed to the outward orientation of the side chain of Asp417. Nonetheless, the mutation might result in a local conformational perturbation by affecting the disposition of the side chain of Arg207, and consequently resulted in a ~10-fold decrease of k_{cat}. In response to the Glu422 mutation, K_M increased 1.4-fold and k_{cat} decreased 3.3-fold. Although Glu422 is not in direct contact with substrate, the Glu422 mutation may affect the enzyme function by affecting the disposition of Lys425.

To better understand the roles played by Glu150 and Glu290, these two residues were further substituted by aspartate, asparagine, and glutamine. Aspartate presumably has a similar ionic state to that of glutamate but with a shorter side chain. On the other hand, glutamine or asparagine might have a chance to substitute partially the function of glutamate if the primary function of the mutated glutamate is to participate in hydrogen bond formations. The kinetic analysis of the respective mutant enzymes (Table 1) shows that the function of Glu150 could be fulfilled by aspartate as evidenced by only 3-fold decrease of k_{cat} in the mutation of Glu150 to Asp. However, neither asparagine nor glutamine could substitute for Glu150. These data suggest that having a negative charge (or the ability of ionization) is the major element for Glu150 to execute its function. Gln150 or Asn150 might induce local conformational changes by affecting the disposition of nearby active-site amino acids, and consequently further damaged the enzymatic activity. In contrast to the results of mutations occurred on Glu150, none of the substitutions could fulfill the function of Glu290, indicating that both the ionic state and the exact spatial position of the carboxylate group in the active site are crucial for catalysis. Taken together with the closeness of the carboxylate group of Glu290 to the C1 carboxylate oxygen of 5-phosphoarabinonate, a general acid–base role is suggested for Glu290.

Previous mutagenesis and affinity labeling studies have suggested that His311 acts as a general base initiating the isomerization reaction, and its ionization is responsible for the activity change on the acidic limb of the activity pH profile [20].

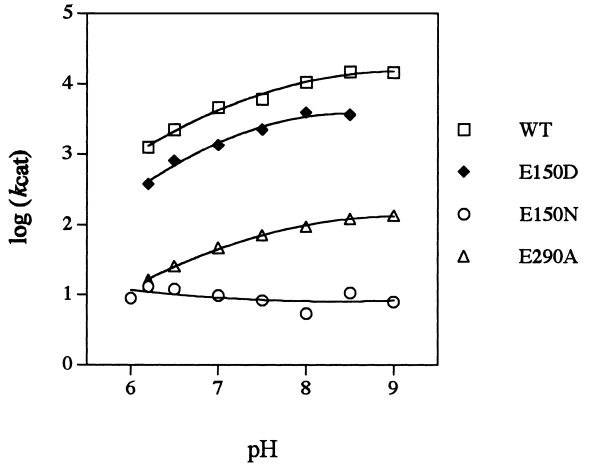

Fig. 2. Effect of pH on the k_{cat} value (min^{-1}) of phosphoglucose isomerase. The activity of variant pgiA was determined at 30°C over the pH range 6–7.5 (imidazole buffer), 8.0–8.5 (Tricine buffer), and 9.0 (glycine buffer).

Glu150, being seated behind His311, seems to provide a negative charge to assist the general-base function of His311 reminiscent of the aspartate–histidine pair of the catalytic triad in serine protease [22]. If this is true, removal of the negative charge from residue 150 would not only cause the reduction of activity but also change the activity pH profile. The V_{max} of several pgiA variants were determined within pH range 6 to 9, and the profiles of log k_{cat} vs. pH are presented in Fig. 2. In wild-type pgiA, the k_{cat} value increased as pH increased. The Glu150 to Asp mutant had a similar pH profile to that of wild-type enzyme; nonetheless, Asn150 could not support the increasing activity at higher pH conditions, indicating that the negative charge on residue 150 has a significant influence on the p*K*a value of His311. In contrast to the mutation of Glu150, removal of a negative charge from residue 290 caused negligible change on the activity pH profile as indicated by the Glu290 to alanine mutation.

The data presented above support our previous hypothesis on the mechanism of isomerization step [13], in which His311 and Glu290 work as a general base–acid pair to prompt the formation of *cis*-enediol intermediate (Fig. 3). By reversing their roles in the subsequent step, the intermediate is then transformed to the product. Through electrostatic interaction, Glu150 could stabilize the partial positive charge developing on the side chain of His311 during the isomerization step. Glu150 also forms a hydrogen bond to His311 and may

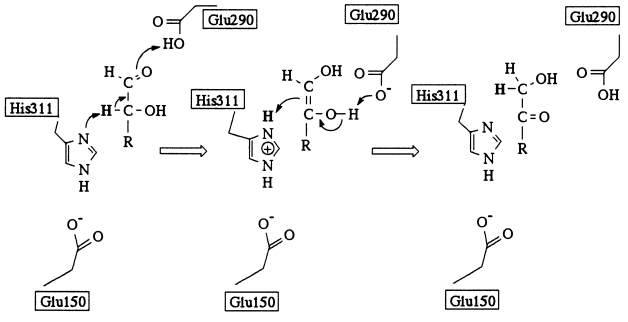

Fig. 3. The roles of Glu150, Glu290, and His311 of pgiA (Glu145, Glu285, and His306 in pgiB) in the catalytic mechanism of the isomerization step.

lock the imidazole ring of His311 in an optimal position for reaction.

3.2. Mutation of the residues around the phosphate group of 5-phosphoarabinonate

According to the crystal structure of pgiB complexed with 5-phosphoarabinonate (Fig. 1), the phosphate group of substrates in transition state may be surrounded by conserved residues Gly205, Gly206, Arg207, Glu290, and Gln418 (Gly200, Gly201, Arg202, Glu285, and Gln413, respectively). To gain more information regarding the substrate recognition mechanism, Gly205, Gly206, and Gln418 were also targeted in the present study. The mutation of Gly206 to leucine resulted in an increase of K_M by a factor of 38, and a decrease of k_{cat}/K_M by a factor of six orders of magnitude (Table 1). The side chain of Leu206 might stick into the active-site cavity and hinder the binding of substrate throughout the catalytic pathway. In contrast to Gly206, mutation of Gly205 caused relatively minor effects on the kinetic constants (Table 1). According to the crystal structure, the side chain of Leu205 would have a great chance of pointing away from the active-site cavity, and this may account for the minor mutational effects. In response to the mutation of Gln418, K_M increased 6-fold and k_{cat}/K_M decreased two orders of magnitude (Table 1) suggesting that Gln418 has an important role for the substrate binding and catalysis. The cavity surrounded by the conserved loop structure Gly205–Gly206–Arg207, Glu290, and Gln418 is thus very important for the accommodations of substrates.

In general, the k_{cat}/K_M value is much more sensitive than K_M in response to mutations in this study. For example, the reduction of the former could reach 5–6 orders of magnitude in some cases, whereas, the most increase of the latter was only $\sim$38-fold. This suggests that the binding energy for the stabilization of ground-state substrate may be attributed cumulatively from all surrounding amino acids so that missing one of them does not cause drastic change in K_M. However, to be an efficient catalyst, all the concerned residues must be present and resume their optimal positions. Deletion of any essential residue or disturbance of the active-site configuration would certainly affect profoundly on the catalytic ability of phosphoglucose isomerase.

Very recently, Jeffery et al. published a crystal structure of rabbit phosphoglucose isomerase complexed with 5-phospho-D-arabinonate [23]. According to the binding position of the inhibitor, they proposed a different mechanism in which the active-site histidine and lysine (corresponding to His311 and Lys425 of pgiA) catalyze the opening of the cyclic form substrates, and the active-site glutamate (corresponding to Glu290 of pgiA) takes the responsibility for the isomerization. Although the ring-opening step is important, isomerization is the rate-limiting step [16,17]. In fact, the spontaneous ring-opening rate constant for fructose 6-phosphate is not slow; it could reach $\sim$19 s^{-1} at 40°C, pH 7.5 [24]. Therefore, the mutations of His311 or Lys425 would not cause severe effects on the overall activity, if these two residues played roles solely in the step of ring opening. From the k_{cat} values of K425A and H311A (0.44 s^{-1} and 0.09 s^{-1} at 30°C, respectively) [19,20], we believe that these two residues must also play significant roles in the step of isomerization. In this study, we also demonstrated the importance of Glu150 that is omitted in the mechanism proposed by Jeffery et al. Although more biochemical and structural data in the future should extend or even modify our catalytic model of phosphoglucose isomerase, the one that we proposed is consistent with most of the current data.

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