Probing the location and function of the conserved histidine residue of phosphoglucose isomerase by using an active site directed inhibitor N-bromoacetyethanolamine phosphate

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Abstract

Phosphoglucose isomerase (EC 5.3.1.9) catalyzes the interconversion of D-glucopyranose-6-phosphate and D-fructofuranose-6-phosphate by promoting an intrahydrogen transfer between C1 and C2. A conserved histidine exists throughout all phosphoglucose isomerases and was hypothesized to be the base catalyzing the isomerization reaction. In the present study, this conserved histidine, His311, of the enzyme from *Bacillus stearothermophilus* was subjected to mutational analysis, and the mutational effect on the inactivation kinetics by N-bromoacetyethanolamine phosphate was investigated. The substitution of His311 with alanine, asparagine, or glutamine resulted in the decrease of activity, in k_{cat}/K_M , by a factor of 10^3 , indicating the importance of this residue. N-bromoacetyethanolamine phosphate inactivated irreversibly the activity of wild-type phosphoglucose isomerase; however, His311 → Ala became resistant to this inhibitor, indicating that His311 is located in the active site and is responsible for the inactivation of the enzyme by this active site-directed inhibitor. The pK_a of His311 was estimated to be 6.31 according to the pH dependence of the inactivation. The proximity of this value with the pK_a value of 6.35, determined from the pH dependence of k_{cat}/K_M , supports a role of His311 as a general base in the catalysis.

Keywords: *Bacillus stearothermophilus*; catalytic mechanism; glucose-6-phosphate isomerase; N-bromoacetyethanolamine phosphate; phosphoglucose isomerases; site-directed mutagenesis

Phosphoglucose isomerase is a multifunctional protein. Besides being a metabolic enzyme that plays a central role in the metabolism of phosphorylated sugar in the glycolysis and gluconeogenesis pathways, it is also identical to neuroleukin (Chaput et al., 1988; Faik et al., 1988), autocrine motility factor (AMF) of tumor cells (Watanabe et al., 1996; Niinaka et al., 1998), and the differentiation and maturation mediator for human myeloid leukemia cells (Xu et al., 1996). The deficiency of this metabolic enzyme affects the metabolism of red blood cells and consequently leads to nonspherocytic hemolytic anemia in humans (Merkle & Pretsch, 1993). Several mutations associated with this hereditary disease have been identified (Beutler et al., 1997; Huppke et al., 1997; Kanno et al., 1998).

As a metabolic enzyme, this protein catalyzes a reversible isomerization between glucose 6-phosphate and fructose 6-phosphate. Numerous kinetic data, including the pH dependence of activity and results of isotope exchange, suggested a base-catalyzed mechanism for the interconversion of aldose and ketose via a cis-enediol intermediate (Rose & O'Connell, 1961; Schray et al., 1973; Malaisse et al., 1990). Following the ring-opening step, a general base with no exchangeable protons, such as a carboxylate or a nonprotonated nitrogen of an imidazole, is thought to transfer stereospecifically a carbon-bound proton of the substrate between C-1 and C-2 (Fig. 1). The values of enthalpy, calculated from the temperature dependence of pK_a s of the pH profile, suggest critical roles of a protonated lysine and a nonprotonated imidazole in the catalysis of the ring-opening and the isomerization step, respectively (Dyson & Noltmann, 1968). Evidence of the involvement of histidine and lysine residues also came from the experiments of affinity labeling (Schnackerz & Noltmann, 1971; Gibson & Gracy, 1980). Chemical modification and affinity labeling also indicated the importance of other amino acids such as arginine (Riordan et al., 1977) and glutamate (O'Connell & Rose, 1973). The im-

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Abbreviations: BrAcNH₂OP, N-bromoacetyethanolamine phosphate; pgi, phosphoglucose isomerase.

Fig. 1. Postulated mechanism via a cis-enediol intermediate for phosphoglucose isomerase.

portance of the conserved lysine and arginine residues was confirmed by mutagenesis (Meng et al., 1998). The properties and the proposed mechanisms of this metabolic enzyme have been reviewed by Rose (1975) and recently by Yüksel and Gracy (1991). Nonetheless, the nature of the active site of the enzyme is poorly understood.

N-bromoacetylanthranilate phosphate (BrAcNHAcOP) was characterized to be an active site-directed inhibitor to mammalian phosphoglucose isomerase in an effort to identify active site residues (Gibson & Gracy, 1980). Peptide mapping technique revealed the linkage of the inhibitor to a histidine residue within sequence of V-L-H*-A-E-N-V. However, this peptide sequence does not occur in any known amino acid sequence of phosphoglucose isomerases ranging from bacteria to mammals. To further establish the identity of the active site histidine, one isozyme of the enzyme from *Bacillus stearothermophilus* (abbreviated as *pgiA*) was subjected to mutational analysis in the present study. The effects of mutations and the inhibition kinetics by BrAcNHAcOP support the active site location of a conserved histidine in phosphoglucose isomerase. This result is consistent with the crystal structure of *pgiB*, another isozyme from *B. stearothermophilus*, which was recently solved (Sun et al., 1999). The structure indicates that the enzyme is a dimer, and the putative active site is composed of the conserved histidine residue from one monomer and those important lysine and arginine residues (Meng et al., 1998) from the adjacent monomer. The probability of being an isomerization-initiating base of this conserved histidine is discussed in this paper.

Results

Determination of the targeted amino acids

The gene of phosphoglucose isomerase has been cloned from a variety of sources ranging from bacteria to mammals over the past decades. The known amino acid sequences of the enzyme were aligned to determine which histidine residues are most probably involved in the catalysis. A simplified alignment scheme is shown in Figure 2. Only one histidine residue is conserved throughout all phosphoglucose isomerases, His311 in *pgiA*. This conserved histidine should be the prime target for mutational analysis. The sequence of V-L-H-A-E-N-V does not occur in all known phosphoglucose isomerases; however, V-L-H exists in the enzyme of several organisms. Although no exact match sequence was found in *pgiA*, a similar sequence V-A-H (residue 29–31) was chosen as the corresponding sequence for mutational analysis. In this study, His311 was substituted with alanine, asparagine, and glutamine; His31 and His32 were substituted with alanine.

Expression and purification of phosphoglucose isomerase

Wild-type and mutant proteins of *pgiA* were expressed in *Escherichia coli* cells and purified to homogeneity (data not shown) according to the procedures described in Materials and methods. Single amino acid substitutions should not bring on significant change in protein structure according to the identical spectra of circular dichroism of wild-type and various mutant enzymes in the far ultraviolet region (data not shown).

Effects of substitutions on steady-state kinetics

The kinetic constants of wild-type and mutant phosphoglucose isomerases are shown in Table 1. The mutation on either His31 or His32 did not cause significant change on kinetic constants, suggesting both residues are irrelevant to catalysis. The mutations on His311 had critical impacts on the enzyme activity. Substitution by alanine resulted in a 1,600-fold decrease in k_{cat} and an insignificant change in K_M relative to that of the wild-type enzyme. The glutamine substitution had a 30-fold decrease in k_{cat} and a 70-fold increase in K_M , leading to a similar effect on k_{cat}/K_M as the alanine substitution. The asparagine substitution also affected k_{cat}/K_M in a same level. According to the transition state theory, the decrease of

	A	B
<i>Bacillus</i> (<i>pgiA</i>)	29VA <u>HH</u> SLHE	307STD <u>LH</u> SLGQYIQEG
<i>Bacillus</i> (<i>pgiB</i>)	28AAHMLHE	302TTDLHSMGQYVQEG
<i>E. coli</i>	96VLHVALRN	382TNGQHAFYQLIHQG
<i>S. cerevisiae</i>	106VYHVALRN	393TNAQHSHFFQLVHQG
<i>Z. mobilis</i>	93VEHMAERG	365TDAQHAVFQLLHQG
<i>A. thaliana</i>	96VLHVALRA	388TNGQHSFYQLIHQG
Mouse	97VLHVALRN	384TNGQHAFYQLIHQG
Human	98VLHVALRN	385TNGQHAFYQLIHQG

Fig. 2. Sequence alignment of partial amino acid sequences of phosphoglucose isomerase. To simplify the alignment scheme, the sequences from only *B. stearothermophilus* (Tao et al., 1989), *E. coli* (Froman et al., 1989), *Saccharomyces cerevisiae* (Tekamp-Olson et al., 1988), *Zymomonas mobilis* (Hesman et al., 1991) *Arabidopsis thaliana* (Thomas et al., 1993), mouse, and human (Faik et al., 1988) are shown. *PgiA* and *pgiB*, sharing 70% homology in overall amino acid sequence, stand for two isozymes of phosphoglucose isomerase from *B. stearothermophilus*. The sequences in block A are similar among most of phosphoglucose isomerases but the enzymes from *B. stearothermophilus*. The residues marked in bold represent the conserved amino acids, and the ones underlined are the residues mutated in the present work.

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

Enzyme	$K_M^{\text{Fructose-6-P}}$ (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M (s ⁻¹ mM ⁻¹)	$\Delta\Delta G^{\ddagger b}$ (kcal/mol)
WT	0.26 ± 0.05	150 ± 10	580	
H31A	0.18 ± 0.05	100 ± 10	560	0.02
H32A	0.28 ± 0.07	130 ± 10	460	0.14
H311A	0.38 ± 0.04	0.09 ± 0.01	0.24	4.70
H311Q	18 ± 1.0	5.0 ± 0.4	0.28	
H311N	2.1 ± 0.3	1.1 ± 0.1	0.52	

^aReactions were carried out in 40 mM potassium phosphate buffer (pH 7.0) at 30 °C.

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

k_{cat}/K_M reflects the magnitude of the loss of binding energy that enzymes employ for the stabilization of the transition state (Fersht, 1985). These mutational results suggest that all three mutations on His311 resulted in approximate amount of loss of binding energy for the stabilization of the transition state. The extensive contribution of His311 on the catalysis and that neither Asn nor Gln compensated the loss of imidazole group of His311 suggest that His311 may not act as simple as a noncharged hydrogen bond acceptor (or donor) in the catalytic pathway.

Kinetics of inactivation of phosphoglucose isomerase by BrAcNHEtOP

Incubation of pgiA with BrAcNHEtOP resulted in a time-dependent irreversible inactivation, and the process followed pseudo-first order kinetics (Fig. 3A). From the inspection of the inactivation rates with different concentrations of BrAcNHEtOP, it is clear that the rates are not directly proportional to the concentration of inhibitor,

indicating that BrAcNHEtOP may bind reversibly to the enzyme prior to inactivation (Lewendon & Shaw, 1993):

The rate of inactivation will approach a maximum value as the inhibitor concentration approaches infinity. A linear expression for the rate has been derived:

$$\tau = \frac{1}{[I]} (T \cdot K_{\text{inact}}) + T \quad (2)$$

in which K_{inact} is $(k_{-1} + k_{+2})/k_{+1}$ and comparable to the Michaelis constant for a substrate, T is the minimum inactivation half-time infinity inhibitor concentration, and τ is the observed inactivation half-time at inhibitor concentration $[I]$ (Hartman et al., 1973). A plot of τ against $1/[I]$ yielded a straight line (Fig. 3B) from which both K_{inact} and T could be determined, and they are 4 mM and 7.6 min, respectively, at pH 7.25. The pseudo-first order rate of inactivation at infinitely concentration of inhibitor (equivalent to k_{+2}) can be calculated by equation $k_{+2} = (\ln 2)/T$. The minimum inactivation half-time of 7.6 min is similar to that of 6.3 and 4.5 min for the inactivation of rabbit and human phosphoglucose isomerase, respectively, by BrAcNHEtOP (Gibson & Gracy, 1980). Glucose 6-phosphate fully protected the enzyme from the inactivation by BrAcNHEtOP (Fig. 4), indicating again that the inhibitor binds to the active site of the enzyme.

Effects of mutations on the inactivation by BrAcNHEtOP

The mutation data suggest that His311 is an active site residue of pgiA. If His311 really resides at the active site, the imidazole of His311 could be the nucleophile attacking BrAcNHEtOP, and consequently causing the inactivation of the enzyme. On the other hand, the mutant enzyme having alanine substitution for His311 would no longer link covalently to BrAcNHEtOP, and therefore

Fig. 3. Inactivation of wild-type phosphoglucose isomerase by BrAcNHEtOP. **A:** 5 μ M of the enzyme (0.25 mg/mL) was incubated with BrAcNHEtOP (0.7–4.8 mM) in 50 mM Tris buffer (pH 7.25) at 30 °C. At each time interval, 0.25 μ g of the enzyme was withdrawn and added into 1 mL standard reaction mixture (see Materials and methods) to assay the residual activity. **B:** Inactivation half-life as a function of the BrAcNHEtOP concentration.

Fig. 4. Effects of mutations and glucose 6-phosphate on the irreversible inactivation of phosphoglucose isomerase by BrAcNHetOP. Enzyme (2.4 mg/mL) was incubated with BrAcNHetOP (8 mM) in 50 mM sodium acetate buffer (pH 6.0) at 30 °C. Glucose 6-phosphate (8 mM) was included in one set of experiments to test the protection effect. An aliquot of reaction mixture was passed, at intervals, through a 5 mL Hi-Trap desalting column (Pharmacia) to separate free molecules of BrAcNHetOP from the enzyme. The enzyme was subsequently assayed for its residual activity. The 100% activity of each set of experiments refers to its own activity before the treatment of BrAcNHetOP.

the inhibitor should not inactivate the enzyme irreversibly. The irreversible inactivation of wild-type and mutant enzymes by BrAcNHetOP is shown in Figure 4. The inactivation kinetics of His31 → Ala mutant enzyme was very similar to that of wild-type enzyme; however, His311 → Ala mutant enzyme was insensitive

to BrAcNHetOP. Since the K_M value of His311 → Ala mutant enzyme for fructose 6-phosphate is close to that of wild-type enzyme (Table 1), the inhibitor should still be able to bind properly to the active site of the mutant enzyme. The reason for the resistance could contribute to the lack of the specific nucleophile in the active site of the mutant enzyme. From the data of Figure 4, we conclude that the imidazole of His311 is the nucleophile responsible for the irreversible inactivation by BrAcNHetOP in wild-type enzyme, and the results substantiate the active site location of His311.

The pK_a value of the imidazole of His311

The temperature dependence of the pH effects on the enzymatic activity suggested a histidine residue acting as a general base to initiate the isomerization reaction (Dyson & Noltmann, 1968). To assess the possibility of His311 to act as a general base, we set out to determine the pK_a value of the imidazole of His311. Since His311 is the nucleophile reacting with BrAcNHetOP, we should be able to determine its pK_a value from the pH dependence of the inactivation of the enzyme by BrAcNHetOP. The inactivation kinetics was carried out over the pH range 6–9, and it was found that the inhibitions all followed pseudo-first order kinetics with saturation of the rate of inactivation in each pH buffer. At least five concentrations of inhibitor were used at each pH to derive the plot of τ against $1/[I]$, from which k_{+2} and K_{inact} values at respective pH were calculated. The pK_a value of the imidazole of His311 was calculated to be approximately 6.31 from the pH dependence of k_{+2}/K_{inact} for wild-type enzyme (Fig. 5A). These data illustrate that the imidazole of His311 is more in nonprotonated than protonated form at physiological pH and is consistent with the hypothesis of being a general base in catalysis. The enzymatic activity of wild-type enzyme was also assayed over the pH range 6–9. Figure 5B shows that k_{cat}/K_M decreases with pH as an ionizable group with an apparent pK_a of 6.35 is protonated. This value is

Fig. 5. Effects of pH. **A:** Effect of pH on the inactivation of wild-type enzyme by BrAcNHetOP. The reaction conditions were as described in the caption of Figure 3. Imidazole was used in pH 6–7.5; while Tris was used in pH 7.0–9. Values of k_{+2}/K_{inact} were calculated as described in the text. The 100% of the second-order rate constant (k_{+2}/K_{inact}) represents $0.0255 \text{ min}^{-1} \text{ mM}^{-1}$. **B:** Effect of pH on the catalytic efficiency (k_{cat}/K_M) of wild-type phosphoglucose isomerase. Reactions occurred in 50 mM buffer solution at 30 °C. Imidazole was used in pH 6–7.5; while Tris was used in pH 7.0–9.

comparable to that derived from the pH dependence of inactivation by BrAcNH₂OP, suggesting that the protonation of His311 may be responsible for the decrease of activity as pH is decreasing.

Discussion

Phosphoglucose isomerase is an intriguing protein, since it not only plays a role in metabolic pathways, but also stimulates the migration of tumor cells and promotes the growth of nerve cells in mammals. Whether these novel functions depend directly on the catalytic ability for phosphosugar is an interesting question. Phosphoglucuronate, a competitive inhibitor of phosphoglucose isomerase, also inhibited the function of tumor autocrine motility factor (Watanabe et al., 1996), suggesting that the availability of the active site is required at least for the action of stimulating the migration of tumor cells. Recently, we found that the enzyme from *B. stearothermophilus* was able to stimulate the movement of mouse colon tumor cells and promoted the growth of nerve cells (Sun et al., 1999). Because there is only 26% similarity in amino acid sequence between the enzyme from *B. stearothermophilus* and mammals, and supposing those similar amino acid segments are located mainly at the active site, such results implicate the likelihood that the catalytic function of phosphoglucose isomerase is involved in those novel functions. Therefore, the understanding of the molecular catalytic mechanism of the enzyme should be physiologically significant.

To elucidate the structure–function relationship of phosphoglucose isomerase, the two isozymes from *B. stearothermophilus* (pgiA and pgiB) have been subjected to crystallographic studies and mutational analysis. In a previous study, the importance of conserved cationic residues, including one arginine and several lysine residues, was analyzed (Meng et al., 1998). In this paper we demonstrate the critical role of the conserved histidine residue (His311 in pgiA) in catalysis and confirm its active site location. The p*K*_a value of His311, calculated from the pH dependence of the inactivation by BrAcNH₂OP, also agrees with the hypothesis that this conserved histidine residue functions as a general base in catalysis. The insignificant role of His31 and His32 in pgiA does not necessarily lead to the conclusion that the histidine in V-A-H sequence in the mammalian enzymes is not important. After all, the alignment in that region was somewhat mandatory. The importance of this particular histidine of the mammalian phosphoglucose isomerase should be evaluated for its own sake.

The kinetic data might be misinterpreted without the guidance of structural data. Recently, the crystal structure of pgiB was solved (Sun et al., 1999). Since the similarity between pgiA and pgiB is 70%, the structure of pgiA should resemble that of pgiB. When we scrutinized the protein structure, we found that all the important conserved cationic residues, indicated by previous mutational data, and the conserved histidine, studied in the present, are located in a cleft in proximity. This cleft is composed of amino acids from two neighboring subunits. The conserved arginine (Arg202) and lysine residues (Lys139, Lys420) are from one subunit, and the conserved histidine (His306, corresponding to His311 in pgiA) is from the other subunit. By combining the kinetic data and the structural information, we conclude that this cleft should be the active site of phosphoglucose isomerase. Nonetheless, the interaction of substrate and the active site amino acids is yet unknown. We are continuing to pursue the crystal structures of enzyme–substrate complex. Until then we can more precisely assign the function of each active site residue, and address the molecular catalytic mechanism of phosphoglucose isomerase.

Materials and methods

Strain, plasmid, and chemicals

E. coli strain DF2145 [(*eda-zwf*)15, *hisG1*(Fs), *pgi*-7::Mu-1, *rpsL115*, *hsdR2*, and *zjj202*::Tn10] was the host for expression of phosphoglucose isomerases from *B. stearothermophilus*. Plasmid pFDI132, which contains the coding gene for pgiA, was kindly provided by Dr. Zujia Sheng (Tao et al., 1989). Glucose-6-phosphate dehydrogenase and β -NAD⁺ were purchased from Sigma (St. Louis, Missouri). BrAcNH₂OP was synthesized according to the protocol described by Hartman et al. (1973). The authenticity and the purity of BrAcNH₂OP were confirmed by NMR.

Site-directed mutagenesis

Site-directed mutations were created according to a polymerase chain reaction (PCR)-based method described previously (Meng et al., 1998). In brief, a pair of 5'-phosphorylated divergent primers were annealed to the desired mutation site of pFDI132. One of the primers contains the mutated sequence at its 5' end. After PCR amplification with *pfu* DNA polymerase (Stratagene, La Jolla, California), the blunt-ended linear DNA product was purified, self-ligated, and transformed back into *E. coli* DF2145. The desired mutation was confirmed by determining the nucleotide sequence with Amplicycles sequencing kit (Perkin-Elmer, Foster City, California).

Production and purification of enzymes

To produce the recombinant phosphoglucose isomerase, *E. coli* DF2145 cells harboring appropriate plasmid were grown overnight at 37 °C in 2 × TY medium (tryptone 16 g/L, yeast extract 10 g/L, NaCl 5 g/L) containing 100 μ g of ampicillin per mL. The culture was then diluted in 1:100 ratio into the same medium and grown at 37 °C for about 15–18 h. Enzymes were purified by modifying the protocol described previously (Hsiao et al., 1997). Briefly, the crude cell extract was incubated at 70 °C for 20 min to precipitate thermo-labile proteins, and the supernatant was fractionated consecutively by phenyl Sepharose CL-4B, DEAE-Sepharose, and Sephacryl S-300 (Pharmacia, Piscataway, New Jersey). Protein concentration was determined by the Coomassie Blue method using the determination kit obtained from Pierce (Rockford, Illinois). Bovine serum albumin (Pierce) was used as a standard.

Enzyme activity determination

The coupled glucose-6-phosphate dehydrogenase method was used to determine the activity of phosphoglucose isomerase (Salas et al., 1965). The standard assay mixture (1 mL) contained 40 mM potassium phosphate (pH 7.0), 10 units of glucose-6-phosphate dehydrogenase, 2 mM β -NAD⁺, and 4 mM 6-phosphofructose. The reaction was initiated by addition of phosphoglucose isomerase, and the reduction of β -NAD⁺ was followed at 30 °C by the increase in absorbance at 340 nm. The blank contained the same reactants with the exclusion of 6-phosphoglucose isomerase. The substrate concentration and the buffer system in the assay mixture were subject to change in the determination of kinetic constants and pH profile. For each reaction condition, assay was repeated at least twice by varying the amount of phosphoglucose isomerase to assure that phosphoglucose isomerase is always the limiting factor.

Determination of kinetic constants and pK_a value

The Lineweaver–Burk plot and the Eadie–Hofstee plot were used to determine K_M and $V_{\max}$. The data were obtained from the average of at least two independent determinations. 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. The K_a of the ionized group responsible for the pH titration curve is determined by using equation $L_H = L - (L_H \times [H^+])/K_a$ (Fersht, 1985).

Circular dichroism

Sample was centrifuged at $28,000 \times g$ for 10 min to remove any precipitated protein, and protein concentration was adjusted to 0.2 mg/mL in 50 mM potassium phosphate buffer (pH 7.0). All spectra were collected in an Aviv 62DS in a 0.1 cm path length cuvette at 25 °C. The time constant of the scan was 10 s/nm.

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