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Characterization of the cupin-type phosphoglucose isomerase from the hyperthermophilic archaeon *Thermococcus litoralis*[1]

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Abstract The gene encoding phosphoglucose isomerase was cloned from *Thermococcus litoralis*, and functionally expressed in *Escherichia coli*. The purified enzyme, a homodimer of 21.5 kDa subunits, was biochemically characterized. The inhibition constants for four competitive inhibitors were determined. The enzyme contained 1.25 mol Fe and 0.24 mol Zn per dimer. The activity was enhanced by the addition of Fe^{2+}, but inhibited by Zn^{2+} and EDTA. Enzymes with mutations in conserved histidine and glutamate residues in their cupin motifs contained no metals, and showed large decreases in k_{cat}. The circular dichroism spectra of the mutant enzymes and the wild type enzyme were essentially the same but with slight differences.

Key words: Phosphoglucose isomerase; Cupin; Metal-binding site; *Thermococcus litoralis*

1. Introduction

Thermococcus litoralis is a typical marine hyperthermophilic archaeon that can grow near the temperature of boiling water, like *Pyrococcus furiosus* [1]. *T. litoralis* is known to anaerobically utilize maltose and cellobiose as carbon and energy sources for a modified Embden–Meyerhof pathway similar to that of many other *Thermococcus* and *Pyrococcus* species [2,3]. Recently, the enzymes for the first three steps (ADP-dependent glucokinase, ADP-dependent phosphofructokinase, and phosphoglucose isomerase [PGI]) of the modified Embden–Meyerhof pathway were discovered in *P. furiosus* [4]. The amino acid sequences of the ADP-dependent kinases of *T. litoralis* showed high identities (58.9% for glucokinase, and 71.6% for phosphofructokinase; J.-J. Jeong, unpublished results) with those reported for *P. furiosus*. The crystal structure of *T. litoralis* ADP-dependent glucokinase revealed that the novel ADP-dependent kinase family exhibits a similar folding to the ATP-dependent ribokinase family [5]. The occurrence of such novel central metabolism is limited to strains of order Thermococcales species such as *T. litoralis* and *P. furiosus*. This suggests that some specific metabolic system may be present in hyperthermophilic marine archaea [6]. Recently, PGI from *P. furiosus* (pfPGI) was purified and biochemically characterized, and then the gene encoding the enzyme was cloned and functionally overexpressed in *Escherichia coli* [7,8]. pfPGI was found to be distantly related to PGIs in general organisms, but rather homologous to the cupin superfamily, which is a group of functionally diverse proteins found in all three life kingdoms, i.e. Archaea, Eubacteria, and Eukaryota [9]. The functions of the cupin superfamily vary, from isomerases and epimerases involved in the modification of cell wall carbohydrates in bacteria to non-enzymatic storage proteins in plant seeds and transcription factors linked to congenital baldness in mammals. In the superfamily, the cupin domain consists of six β-strands within a conserved β-structure. Most cupin enzymes are metalloenzymes with two conserved sequences (consensus, $G(x)_5HxH(x)_{3,4}E(x)_6G$, motif 1; and $G(x)_5PxG(x)_2H(x)_3N$, motif 2). It has been confirmed that the two His residues and the Glu residue in motif 1 together with the His residue in motif 2 act as ligands for the binding of an active-site metal ion, such as Fe, Mn, or Zn [10].

In this paper, we describe the cloning of the PGI gene from *T. litoralis*, and characterization of the enzyme (tlPGI) produced by *E. coli*. Enzymes with mutations in the conserved metal-binding site were characterized to investigate the importance of a metal ion for its structure and activity. This is the first report concerning the characteristics of the metal-binding site of an archaeal cupin-type PGI.

2. Materials and methods

2.1. Preparation of a DNA probe by means of PCR and Southern hybridization

The genomic DNA of *T. litoralis* was prepared as described previously [11]. Sense and antisense primers were synthesized based on the strictly conserved amino acid sequences of known PGIs: VRRLSDM and DYGTIAE (residues 25–31 and 160–166 of tlPGI, respectively). Information on the preliminarily determined nucleotide sequence of *T. litoralis* PGI (I. Yoshioka and S. Koga, personal communication) was referred to for the design of the primers. The two primers used for polymerase chain reaction (PCR) were 5′-GTT AGA AGG CTC AGC GAC ATG-3′ and 5′-TTC GGC GAT TGT TCC ATA GTC-3′. PCR amplification was conducted with Ex Taq DNA polymerase (Takara) and a PCR Thermal Cycler PERSONAL (Takara). A PCR product of about 430 bp was amplified using *T. litoralis* chromosomal

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[1] Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession number AB081724.

Abbreviations: PGI, phosphoglucose isomerase; tlPGI, phosphoglucose isomerase from *Thermococcus litoralis*; pfPGI, phosphoglucose isomerase from *Pyrococcus furiosus*; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; ORF, open reading frame; ICPAES, inductively coupled plasma atomic emission spectroscopy

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DNA as a template and used as a hybridization probe after it had been labeled with digoxigenin (DIG). For Southern hybridization, chromosomal DNA from *T. litoralis* was digested with appropriate restriction enzymes (*Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, and *Xho*I), separated on a 0.8% agarose gel, and then blotted onto a Hybond N^+ membrane (Qiagen). Southern hybridization was performed at 68°C with oligonucleotides (100 pmol) labeled with a DIG oligonucleotide tailing kit (Roche Applied Science). The membrane was subsequently washed twice with 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) for 5 min, and twice with 0.1×SSC, 0.1% SDS for 5 min. Southern hybridization showed that an about 3.4-kb *Pst*I digest hybridized with the probe.

2.2. Cloning of the tlPGI gene from T. litoralis

Chromosomal DNA of *T. litoralis* was partially digested with *Pst*I. DNA fragments ranging in size from 0.5 to 4 kb were purified from agarose gels and subsequently ligated into the *Pst*I site of pUC119. *E. coli* JM109 was transformed with each ligation mixture. A 3.4-kb *Pst*I fragment was labeled with the DIG oligonucleotide probe. Positive clones were analyzed by restriction analysis and sequencing. Sequence analysis was performed with a CEQ 2000XL DNA Analysis System (Beckman Coulter). The following primer set was designed to amplify the open reading frame (ORF) by PCR: 5′-CGG GGG TGA ACA TAT GAA GTA TAA GG-3′ (sense; underlined, *Nco*I site), and 5′-CCT TTT TCT CGA GTT TGA AAG-3′ (antisense; underlined, *Xho*I site). The PCR product was digested and cloned into the *Nco*I/*Xho*I-digested pET21d vector.

2.3. Overexpression of the tlPGI gene in E. coli

An overnight culture of *E. coli* BL21 (DE3) CodonPlus RIL harboring the PGI expression vector was used as a 1% inoculum for 1 l of Luria–Bertani medium containing 100 μg/ml ampicillin. Gene expression was induced by adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at an optical density of 0.6 at 600 nm. Growth was allowed to continue for 6 h at 37°C, and cells were harvested by centrifugation (10 000×*g* for 30 min), resuspended in 50 ml of 20 mM Tris–HCl buffer, pH 7.5, and then sonicated for 15 min on ice until near complete lysis was achieved. Cell debris was removed by centrifugation (10 000×*g* for 30 min), and the supernatant was treated at 90°C for 20 min to denature *E. coli* proteins. The precipitated protein was removed by centrifugation (12 000×*g* for 30 min). The resulting supernatant was used for further purification.

2.4. Purification of the recombinant tlPGI

The supernatant was filtered through a 0.22-μm filter and then applied to a hydroxyapatite (Bio-Rad) column that had been equilibrated with 20 mM phosphate-NaOH, pH 6.8. Bound proteins were eluted with a linear gradient of phosphate-NaOH buffer (20–400 mM). The active fractions were concentrated with a Centriprep (Millipore). The concentrated protein was loaded onto a Mono-Q HR 5/5 column (Amersham Biosciences) equilibrated with 20 mM Tris–HCl, pH 7.5. The PGI activity was eluted with a linear gradient of NaCl (0–0.5 M in Tris–HCl, buffer). Then, the concentrated protein was loaded onto a Superdex 200 HiLoad 26/60 column (Amersham Biosciences) equilibrated with 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, and eluted with the same buffer. The purified enzyme was desalted and concentrated with 20 mM Tris–HCl, pH 7.5, using a Centriprep.

2.5. Determination of the enzyme activity

The formation of glucose 6-phosphate (G-6-P) from fructose 6-phosphate (F-6-P) was determined by measuring the formation of NADPH at 340 nm (standard method for measurement of PGI activity, but the reverse for glycolysis). The assay mixture comprised 100 mM Tris–HCl, pH 7.5, 2 mM F-6-P, 10 mM $MgCl_2$, 0.5 mM NADP, and 1.75 U of yeast D-glucose-6-phosphate dehydrogenase (Sigma). The formation of F-6-P from G-6-P (reverse direction to the standard method) was determined by measuring NADH oxidation at 340 nm. The assay mixture comprised 100 mM Tris–HCl, pH 7.5, 20 mM G-6-P, 2.5 mM ADP, 5 mM $MgCl_2$, 0.2 mM NADH, 1 U of recombinant ADP-dependent phosphofructokinase from *T. litoralis* (prepared by J.-J. Jeong, unpublished result), 0.23 U of aldolase from rabbit muscle (Sigma), and 11 U of glycerophosphate dehydrogenase-triosephosphate isomerase from rabbit muscle (Sigma). The assay was performed at 50°C. One unit was defined at the amount of enzyme required to convert 1 μmol of F-6-P or G-6-P per minute.

2.6. Kinetic parameters

Kinetic parameters were determined at 50°C with standard assay mixtures containing various concentrations of the substrates. For determination of the K_m and V_{max} values for F-6-P and G-6-P, the concentration of the substrate ranged from 0.05 to 5 mM, and 0.5–20 mM was used.

2.7. Inhibitors of PGI activity

Possible inhibitors were tested by means of the standard PGI assay at 50°C with F-6-P as the substrate. Fructose, glucose, mannose, galactose (10 mM), acetyl phosphate, phosphoenolpyruvate (10 mM), AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, or UTP (3.5 mM) was added to the standard assay mixture. To investigate the effects of cations and a chelator, 10 mM NaCl, KCl, $MnCl_2$, $CoCl_2$, $NiCl_2$, $ZnCl_2$, $CdCl_2$, $CuCl_2$, or EDTA was used instead of 10 mM $MgCl_2$ in the standard assay mixture. In the presence of 10 mM $FeCl_2$, the assay was performed at 37°C and the activity was compared with that under the standard conditions at the same temperature. The inhibition constants for fructose 1-phosphate, fructose 1,6-bisphosphate, mannose 6-phosphate, and gluconate 6-phosphate were determined by means of Dixon plots [12], measuring the activity with various inhibitor concentrations (0.25–3.0 mM for fructose 1-phosphate, 2.0–24 mM for fructose 1,6-bisphosphate, 0.25–3.0 mM for mannose 6-phosphate, and 0.025–0.3 mM for gluconate 6-phosphate) at two substrate concentrations (1.0 and 10 mM F-6-P).

2.8. Metal quantification

Inductively coupled plasma atomic emission spectroscopy (ICPAES) (SPS-1200 VR; Seiko Instrument, Chiba, Japan) was performed to determine the metal species bound to the purified protein. 17 mg/ml of PGI was used, and the amounts of metals were calculated using appropriate standard solutions.

2.9. Site-directed mutagenesis

Mutant tlPGIs were constructed with a 'QuickChange' site-directed mutagenesis kit (Stratagene) using the following and complementary oligonucleotides. Oligonucleotides 5′-GAC CAA AGG CGC CTA TGC TTC GAA AAT AG-3′, 5′-GGC CAC TAT GCT TCG AAA ATA GAC AGG-3′, 5′-CAC TAT CAT TCG AAA ATA GAC AGG GCA GTG GTC TAT TTT G-3′, and 5′-GCA CTA TAG TTT ACG TAC CTC CAT ACT GGG CTG CCA GAA C-3′ were used to obtain mutant enzymes H89A, H91A, E98V, and H137A, respectively. The mutations were confirmed by determining the nucleotide sequences. Mutant enzymes were expressed and purified by the same method as that for the wild-type enzyme. The heat treatment step was performed at 90°C for 5 min, and only a hydroxyapatite column was needed to purify the mutant enzymes to homogeneity.

2.10. Circular dichroism (CD) measurements

CD spectra were obtained with a Jasco spectropolarimeter J-720 (Jasco, Tokyo) with a 0.1-cm path length cuvette at 25°C. Protein samples were dissolved in 10 mM sodium phosphate buffer (pH 6.8) at 50 μM concentration.

3. Results and discussion

3.1. Cloning of the PGI gene

As described above, we screened a *T. litoralis Pst*I library constructed with pUC119 by colony hybridization, and obtained a clone containing the entire tlPGI gene. The region containing the entire tlPGI gene was sequenced for both strands. The coding region for the tlPGI gene comprised 570 bp, and was preceded by a transcription factor B recognition element (BRE site), a putative TATA box element, and a ribosome-binding site, as observed for *Pyrococcus* PGI genes [8]. Therefore, transcription of the tlPGI gene would be regulated by a similar mode as that of the pfPGI gene. The ORF encoded a polypeptide of 190 amino acid residues (21.5 kDa). The amino acid sequence of tlPGI was very similar to those of PGIs from *Pyrococci* (84%, 81.3%, and 82.9%

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Tl pgi      1  --MKYKEPFGVKLDFETGIIENAKKSVRRLSDMKGYFIDEEAWKKMVEEGDPVVYEVYAIEQEEKEGDLNFATTVL    74
Pf pgi      1  ---MYKEPFGVKVDFETGIIEGAKKSVRRLSDMEGYFVDERAWKELVEKEDPVVYEVYAVEQEEKEGDLNFATTVL    73
Ph pgi      1  MVKMYKEPLGVKVDFESGVIEGAKKLVRRLSDMKGYFLDEETWRELVEREDPVVYEVYAVEQEEKEGDLNFATTVL    76
Pa pgi      1  ---MYKEPLGVKVDFNTGVIPGAKKIVRRLSDMKGYFLDEKSWEELVKKEDPIVYEVYAIEQEEKEGDLNFATTIL    73

                          Motif 1                                     Motif 2
                     _______________________                    ______________
                            * *      *                                  *
Tl pgi     75 YPGKVGNEFFMTKGHYHSKIDRAEVYFALKGKGGMLLQTPE---GEARFIEMEPGTIVYVPPYWAHRTINTGDKPFI   148
Pf pgi     74 YPGKVGKEFFFTKGHFHAKLDRAEVYVALKGKGGMLLQTPE---GDAKWISMEPGTVVYVPPYWAHRTVNTGDEPFI   147
Ph pgi     77 YPGKVGKEFFFTKGHFHAKRDRAEVYIALKGKGGMLLQTPE---GEARWIPMEPGTVVYVPPYWAHRTVNTGDEPFI   150
Pa pgi     74 YPGKVGKEFFFTKGHYHSKKDRAEVYVALKGKGGMLLQTPE---GEARWIPMEPGTVVYVPPYWAHRTVNTGDEPFI   147
MJ1618              GSK----TLHKHYT--SEEIYYILEGRGLMT--------LDNEKFEVKKGDTIYIPPKTPHKIEN
S.meliloti          GDEYFMTRGHIHARPNRPEMYYGEAGVGVMLLESPH---GEIRTIEMRARTMCYVPPFWIHRSVN
SYN_PMI             GHR---LSLQMHH--HRSEHWIVVSGTARIVCG--------DKEELLEPNQSTYVPQCTAHRLEN
HV_GLP              GGT---NPPHIHPRA--TEIGMVMKGELLVGILGSLDSGNKLYSRVVRAGETFVIPRGLMHFQFN
                     ------->          ------> ----->          ----->  --->     --->
                        C                 D      E                F      G         H

Tl pgi    149 FLALYPADAGHDYGTIAEKGFSKIVVEENGKVVVKDNPKWRM                                   190
Pf pgi    148 FLAIYPADAGHDYGTIAEKGFSKIVIEENGEVKVVDNPRWKK                                   189
Ph pgi    151 FLAIYPADAGHDYGTIAEKGFSKIVIEENGEVKVVDNPRWKN                                   192
Pa pgi    148 FLAIYPADAGHDYGTIAERGFSKIVIEENGEVKVVDNPRWKS                                   189
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Fig. 1. Multiple sequence alignment of deduced amino acid sequences of archaeal cupin-type PGIs and selected cupin proteins. The abbreviations are as follows: Tl pgi, PGI from *T. litoralis* (AB081724); Pf pgi, PGI from *P. furiosus* (AF381250); Ph pgi, putative PGI from *P. horikoshii* (PH1956, AP000007); Pa pgi, putative PGI from *P. abyssi* (PAB1199, AJ248228); MJ1618, hypothetical protein from *Methanococcus jannaschii* (g1499583 g1592216); S. meliloti, hypothetical protein from *Sinorhizobium meliloti*; SYN_PMI, phosphomannose isomerase from *Synechocystis* sp.; and HV_GLP, germin-like protein from *Hordeum vulgare* (barley). Conserved histidine and glutamic acid residues are indicated by asterisks. The designation of the six strands (C–H) is according to the notation developed for the vicilin phaseolin [18]. Regions of motifs 1 and 2 of the cupin domain are also indicated above.

identity with *P. furiosus*, *P. abyssi*, and *P. horikoshii*, respectively; Fig. 1).

3.2. Functional overexpression and purification of the tlPGI gene from E. coli

The function of the ORF was proved by overexpression in *E. coli*. The ORF was amplified by PCR, and then cloned into vector pET21d and transformed into *E. coli* BL21 (DE3) CodonPlus RIL. SDS–PAGE analysis of a heat-treated cell-free extract revealed an additional band corresponding to approximately 26.7 kDa, which corresponded to the calculated molecular mass of the gene product. The recombinant tlPGI was purified about 6.9-fold by heat treatment and chromatography on hydroxyapatite, Mono-Q, and Superdex 200 HiLoad 26/60 columns. Yield of activity was 35%. The purified protein was electrophoretically homogeneous as judged on denaturing SDS–PAGE (Fig. 2). Additional native PAGE analysis gave a single protein band (data not shown). The purified enzyme showed specific activity of 47.3 U/mg in the direction of G-6-P formation at 50°C and pH 7.5. The apparent molecular mass of native tlPGI was determined to be approximately 39 kDa, indicating a homodimeric structure for the native enzyme, like for pfPGI [7,8].

3.3. Catalytic properties of the recombinant tlPGI

The recombinant tlPGI showed Michaelis–Menten kinetics at 50°C, with K_m values of 11.7 and 1.7 mM for G-6-P and F-6-P, and V_{max} values of 45.5 and 72.6 U/mg for G-6-P and F-6-P, respectively. The K_m and V_{max} values of tlPGI were not greatly different from reported values for pfPGI [7,8]. The effects of potential inhibitors on the activity of the recombinant PGI were examined by means of the standard assay with F-6-P as the substrate. The addition of fructose, glucose, mannose, galactose (10 mM), acetyl phosphate, phosphoenolpyruvate (10 mM), AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, or UTP (3.5 mM) did not have any effect on the PGI activity. The standard assay conditions for this study with F-6-P as the substrate included 10 mM $MgCl_2$. An assortment of metal ions (10 mM) and a chelator (10 mM EDTA), instead of Mg^{2+}, were examined for their ability to either enhance or inhibit PGI activity. The activity at 50°C was not greatly affected by Na^+ (100%), K^+ (82%), or Mn^{2+} (82%), but was inhibited by Co^{2+} (41%), Ni^{2+} (16%), Zn^{2+} (11%), Cd^{2+} (4.4%), Cu^{2+} (3.8%), and EDTA (22%). In order to prevent the oxidation of ferric ion, the activity with 10 mM Fe^{2+} was measured at 37°C, and it showed 156% enhancement compared with the control activity. The activity of the tlPGI is inhibited by classical PGI inhibitors such as mannose 6-phosphate, fructose 1-phosphate, and fructose 1,6-bisphosphate [13]. Similar inhibition characteristics of pfPGI with these compounds were reported by Verhees et al. [8]. To elucidate the characteristics of the inhibition in more detail, we determined the inhibition modes and constants (K_i) for these compounds with F-6-P as the substrate. All four compounds

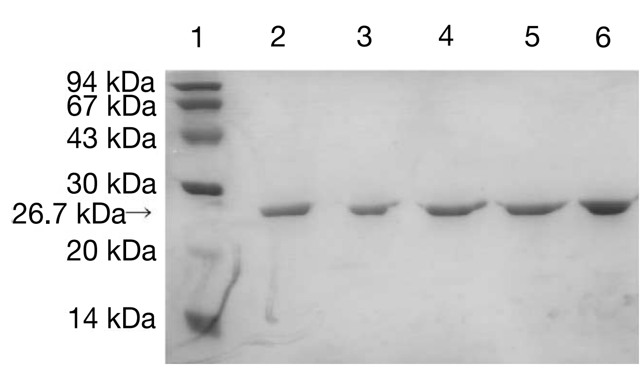

Fig. 2. SDS–PAGE (15%) of the purified wild-type and mutant tlPGIs. Molecular weight standards (lane 1), wild-type tlPGI (lane 2), H89A (lane 3), H91A (lane 4), E98V (lane 5), and H137A (lane 6). Lanes were visualized with Coomassie brilliant blue.

Table 1
Kinetic parameters[a] and metal contents of the wild-type and mutant tlPGI

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	Fe (mol/dimer)	Zn (mol/dimer)
In the direction of F-6-P formation					
Wild-type	11.7	42	3.5	1.25	0.240
In the direction of G-6-P formation					
Wild-type	1.7	26	16	1.25	0.240
H89A	3.5	0.021	0.0059	ND[b]	ND
H91A	2.1	0.50	0.24	ND	ND
E98V	0.59	0.00030	0.00051	ND	0.040
H137A	1.4	0.34	0.24	ND	ND

[a]Enzyme activity was measured at 50°C.
[b]ND, not detectable (< 0.010).

were competitive inhibitors of tlPGI, and the K_i values for fructose 1-phosphate, fructose 1,6-bisphosphate, mannose 6-phosphate, and gluconate 6-phosphate were determined to be 4.0, 12, 2.3, and 0.38 mM, respectively. The K_i value of tlGK for gluconate 6-phosphate is not greatly different from those (0.12–0.48 mM) of conventional PGIs [14], indicating that the mode of recognition at the substrate-binding site of cupin-type PGIs may be similar to that of conventional PGIs, despite the fact that they belong to completely different protein superfamilies.

3.4. Metal-binding site of tlPGI

The characteristic cupin domain comprises two conserved motifs (Fig. 1), each corresponding to two β-strands, separated by a less conserved region composed of another two β-strands with an intervening loop of variable length [9]. In metal-containing cupins, the two His residues and the Glu residue in motif 1, and the His residue in motif 2 bind a metal ion, such as Fe, Mn, or Zn (summarized in [10]).

An absorption spectrum of the recombinant tlPGI showed an absorption peak at 418.5 nm (Fig. 3). In order to identify the metal ion, we carried out ICPAES, which revealed that the recombinant tlPGI contains 1.25 Fe atoms and 0.24 Zn atoms per dimer. Therefore, about 63% of the monomers contain an iron atom on average. In the crystal structure of tlPGI with two subunits in the asymmetric unit (J.-J. Jeong, unpublished data), the iron site at one subunit showed a strong electron density peak, but a peak at another subunit was very weak. When the crystal was soaked with 2 mM $FeSO_4$, both sites gave strong peaks. On the other hand, Ca, Ni, Mg, Co, Cu, Mn, Mo, and Cd atoms were not detected at all or at very low levels (< 0.062 mol per tlPGI dimer). We assume that tlPGI is an iron-dependent enzyme, because its activity increases on addition of the Fe^{2+} ion, but is decreased by the Zn^{2+} ion or EDTA, as described above.

Four mutant enzymes, H89A, H91A, H137A and E98V, were constructed to determine which are important for metal binding. All of the mutant enzymes were expressed in *E. coli* and purified with a hydroxyapatite column. SDS–PAGE of the purified mutant enzymes indicated that all were essentially homogeneous (Fig. 2). The absorption maximum at 420 nm as found in wild-type enzyme as isolated (Fig. 3) was completely lost in every mutant enzyme. The kinetic parameters of the mutant enzymes were determined and compared with those of the wild-type tlPGI, as summarized in Table 1. All mutants showed a 520- to 87 000-fold decrease in k_{cat} compared with the wild-type enzyme. ICPAES revealed that all four mutants contain no Fe or Zn (Table 1), or other metals (< 0.042 mol per tlPGI dimer).

Far-UV CD spectra of the wild-type and mutant enzymes were obtained to determine whether or not these proteins are properly folded (Fig. 4). Since tlPGI, which is a member of the cupin protein superfamily, is expected to be a β-sheet-rich protein, the CD spectra were not characteristic of a helical conformation, which exhibits two minima at 208 and 222 nm, and an isodichronic point at 200 nm. Although secondary structure prediction did not provide any reliable results, comparison of these spectra clearly indicates that the mutant pro-

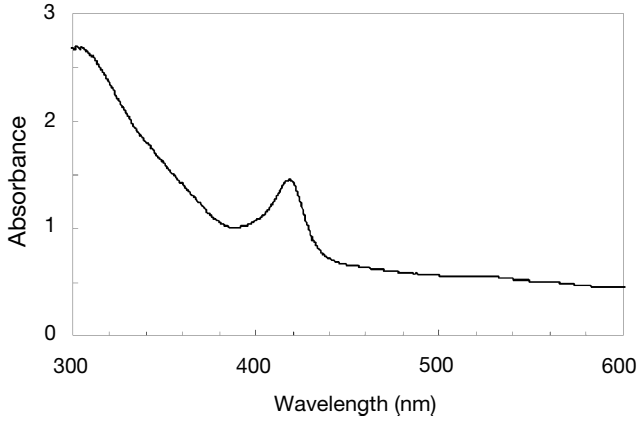

Fig. 3. Absorption spectrum of wild-type recombinant tlPGI as isolated. The enzyme concentration was 10 mg/ml in 20 mM Tris–HCl, pH 7.5.

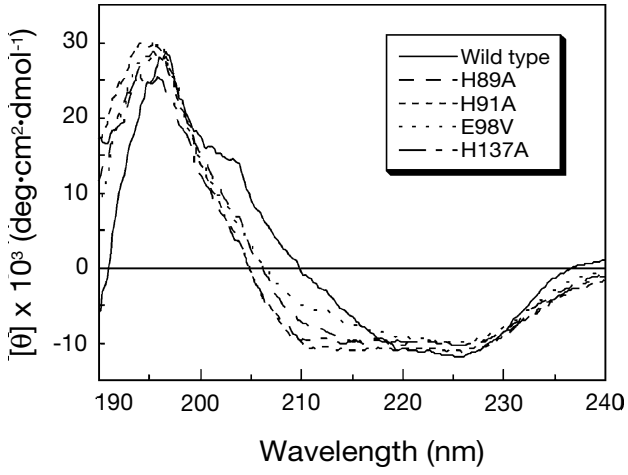

Fig. 4. CD spectra of the wild-type and mutant tlPGIs.

teins have essentially similar, but measurably different spectra compared to the wild-type enzyme.

Combining with the results indicated above, we concluded that the iron atom of tlPGI is essential for its isomerase activity. Moreover, all four conserved residues in the cupin motifs are required for iron binding, and the iron atom at the center of the cupin barrel is required for maintaining the exact folding of the enzyme, but not for its global folding. A multistep catalytic mechanism of conventional PGIs was established on the basis of extensive biochemical characterization, and crystal structures complexed with various inhibitors and substrates [15,16]. Important residues for catalysis have been identified to be His, Lys, Glu, and Arg, but no metal ion is involved. Although the recognition of a substrate at the active site of cupin-type and conventional PGIs may be similar at a certain level, the catalytically important elements should be completely different. On the other hand, phosphomannose isomerase from *Candida albicans*, which is a member of the cupin superfamily, contains an essential zinc ion in the active site [17]. This zinc ion is liganded by Gln, His, and Glu residues in motif 1, and by a His residue in motif 2. The catalytic mechanism of phosphomannose isomerase, however, remains to be elucidated. In spite of the difference in the essential metal ion (iron or zinc) and the first metal-ligand residue in motif 1 (His or Gln), archaeal cupin-type PGIs and phosphomannose isomerases may have a common catalytic mechanism for phosphorylated sugar isomerase activity.

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