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SHORT COMMUNICATION

Alteration of the substrate specificity of *Thermus caldophilus* ADP-glucose pyrophosphorylase by random mutagenesis through error-prone polymerase chain reaction

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Abstract Expanding the scope of stereoselectivity is of current interest in enzyme catalysis. In this study, using error-prone polymerase chain reaction (PCR), a thermostable adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase) from *Thermus caldophilus* GK-24 has been altered to improve its catalytic activity toward enatiomeric substrates including [glucose-1-phosphate (G-1-P) + uridine triphosphate (UTP)] and [*N*-acetylglucosamine-1-phosphate (GlcNAc) + UTP] to produce uridine diphosphate (UDP)-glucose and UDP-*N*-acetylglucosamine, respectively. To elucidate the amino acids responsible for catalytic activity, screening for UDP-glucose pyrophosphorylase (UGPase) and UDP-*N*-acetylglucosamine pyrophosphorylase (UNGPase) activities was carried out. Among 656 colonies, two colonies showed UGPase activities and three colonies for UNGPase activities. DNA sequence analyses and enzyme assays showed that two mutant clones (H145G) specifically have an UGPase activity, indicating that the changed glycine residue from histidine has the base specificity for UTP. Also, three double mutants (H145G/A325V) showed a UNGPase, and A325 was associated with sugar binding, conferring the specificity for the sugar substrates and V325 of the mutant appears to be indirectly involved in the binding of the *N*-acetylamine group of *N*-acetylglucosmine-1-phosphate.

Keywords ADP-glucose pyrophosphorylase (ATP: α-glucose-1-phosphate adenylytransferase, EC 2.7.7.27) · *Thermus caldophilus* GK-24 · Error-prone polymerase chain reaction

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Introduction

The unique character of the extremely thermostable enzyme system, to catalyze *in vitro* the synthesis of nucleotide-sugars under appropriate conditions, can be exploited for the enzymatic synthesis of carbohydrates [1–3]. For example, the potential utilization of sugar-nucleotide pyrophosphorylases from *Thermus caldophilus* GK-24 has been applied for the enzymatic synthesis of activated sugars such as adenosine diphosphate (ADP)-glucose, uridine 5′-diphosphate (UDP)-glucose, UDP-*N*-acetylglucosamine (GlcNAc) and deoxythymidine diphosphate (dTDP)-L-rhamnose [1–4].

ADP-glucose pyrophosphorylase (ATP:α-glucose-1-phosphate adenylytransferase, EC 2.7.7.27; AGPase) is a major regulatory enzyme in the biosynthesis of α-glucans in bacteria and plants [5–7]. This enzyme catalyzes the reversible synthesis of ADP-glucose which serves as a glucosyl donor and PPi from ATP and glucose-1-phosphate (G-1-P) in the presence of a divalent metal ion. Its regulatory and catalytic properties from several sources have been extensively reviewed by Preiss *et al*. [8]. It is generally thought that AGPase is commonly modulated by

Table 1 Oligonucleotides employed in this study

Oligonucleotides	Sequence
1	5′-CCCGTCT**GGTACC**GGGGCACGGCG
2	5′-GAAGCCCC**GGGCCC**GGTCCAGCTC

allosteric effectors and exists in a tetrameric structure [7]. However, much difference in the allosteric properties and structure of the protein is seen between the higher plant and bacterial enzymes [5, 6, 8]. The enzyme from bacteria is a homotetramer with a molecular mass of 200 kDa, encoded by a single gene locus [7], while the higher plant enzymes have a more complex heterotetrameric structure with two dissimilar subunits [9–16]. Several groups have identified the amino acids associated with the substrate specificity and the regulatory properties through chemical modification or site-directed mutagenesis. In *Anabaena* sp., for example, the modification studies using pyridoxal-5-phosphate (PLP) indicated that K419, which is homologous to K440 and K441 in the small subunits of the spinach and potato tuber enzymes, is located with the allosteric regulatory sites. In *Escherichia coli*, K195 turned out to be specifically involved in the binding of G-1-P [17].

On the other hand, chemical modification and substrate analogue studies of *E. coli* AGPase suggest that both lysine and arginine are important for enzyme activity [18–20]. In addition, a number of other sites have been assigned in AGPase [21] in *E. coli*: The N-terminal region [22], G336 [23], and P295 [24], represent known allosteric activation or inhibition sites in *E. coli* AGPase. The AGPase from *E. coli*, which was subjected to site-directed mutagenesis of the G336 showed a higher apparent affinity for the substrates, and the P295 showed a lower apparent affinity for the inhibitor, AMP [23, 24].

In the previous papers, we reported on the purification, characterization and molecular cloning of AGPase from *T.caldophilus* GK-24 [1, 25]. From the molecular weight and the primary structure, the wild-typed AGPase has a high similarity (an identity of 48%) to the AGPases from *E. coli* and *Salmonella typhimurium*, but the nature of its allosteric regulation is quite different from that of the bacterial enzyme; the modification of lysine with PLP increased the AGPase activity from *E. coli* and spinach but decreased that from *T. caldophilus* GK-24. Likewise, phenylglyoxal-induced modification of the arginine residue decreased the AGPase activity from *E. coli* and spinach but increased that from *T. caldophilus* GK-24 [18, 19, 20, 26]. For arginine residue-related activation, both the *Agrobacterium tumefaciens* enzyme [27] and the *Rhodobacter sphaeroides* enzyme [28] were known to be activated by modification with arginyl reagents, although the tendencies are depending on the assay conditions.

In this study, error-prone PCR was performed to determine the amino acids involved in the substrate-specific AGPase activity by screening for UDP-glucose (UGPase) and UDP-GlcNAc pyrophosphorylase (UNGPase) activity. Among 656 colonies, two colonies showed UGPase activity and three colonies for UNGPase activity. The DNA sequence analysis indicated that H145 is critical for ATP-binding and A325 for G-1-P binding.

Materials and methods

General procedures

Restriction enzyme digestion, transformation, and other standard molecular biology techniques were carried out as described by Sambrook *et al*. [29].

Microorganism, cultivation, DNA isolation, PCR, cloning and expression of AGPase gene in *E. coli*

T. caldophilus GK-24 cells [1, 25] was grown and 2 μg of the *T. caldophilus* GK-24 genomic DNA was restricted for Southern blotting. The digested DNA was resolved on a 1% agarose gel and was transferred to a positively-charged nylon membrane (Boehringer Mannheim, Germany) by a capillary method. In order to clone the AGPase gene from the *T. caldophilus* GK-24, the protein sequences of N-terminal (VEVLGMI) and internal region (MALASMG) obtained from our previous study [1], was reverse-translat-

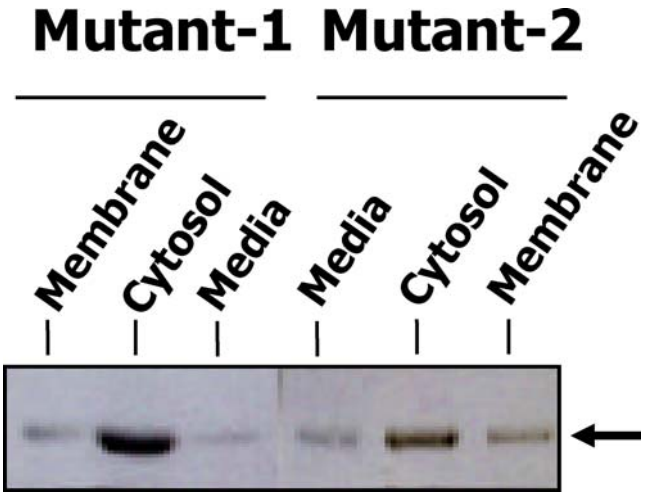

Fig. 1 Expression of *AGPase* gene and immunoblots of the mutant-1 and mutant-2 enzymes. Each recombinant *E. coli* DH5α/pHCE-UGP and *E. coli* DH5α/pHCE-UNGP was cultured for 18 h and each fraction (10 μg) such as culture medium, membrane fraction and cytosolic fractions was resolved on a 12% SDS-polyacrylamide gel and then transferred to a nitrocellulose filter. The filter was incubated with antisera against *T. caldophilus* GK-24 AGPase. Arrow indicates the position of the mutant-1 and mutant-2 enzymes

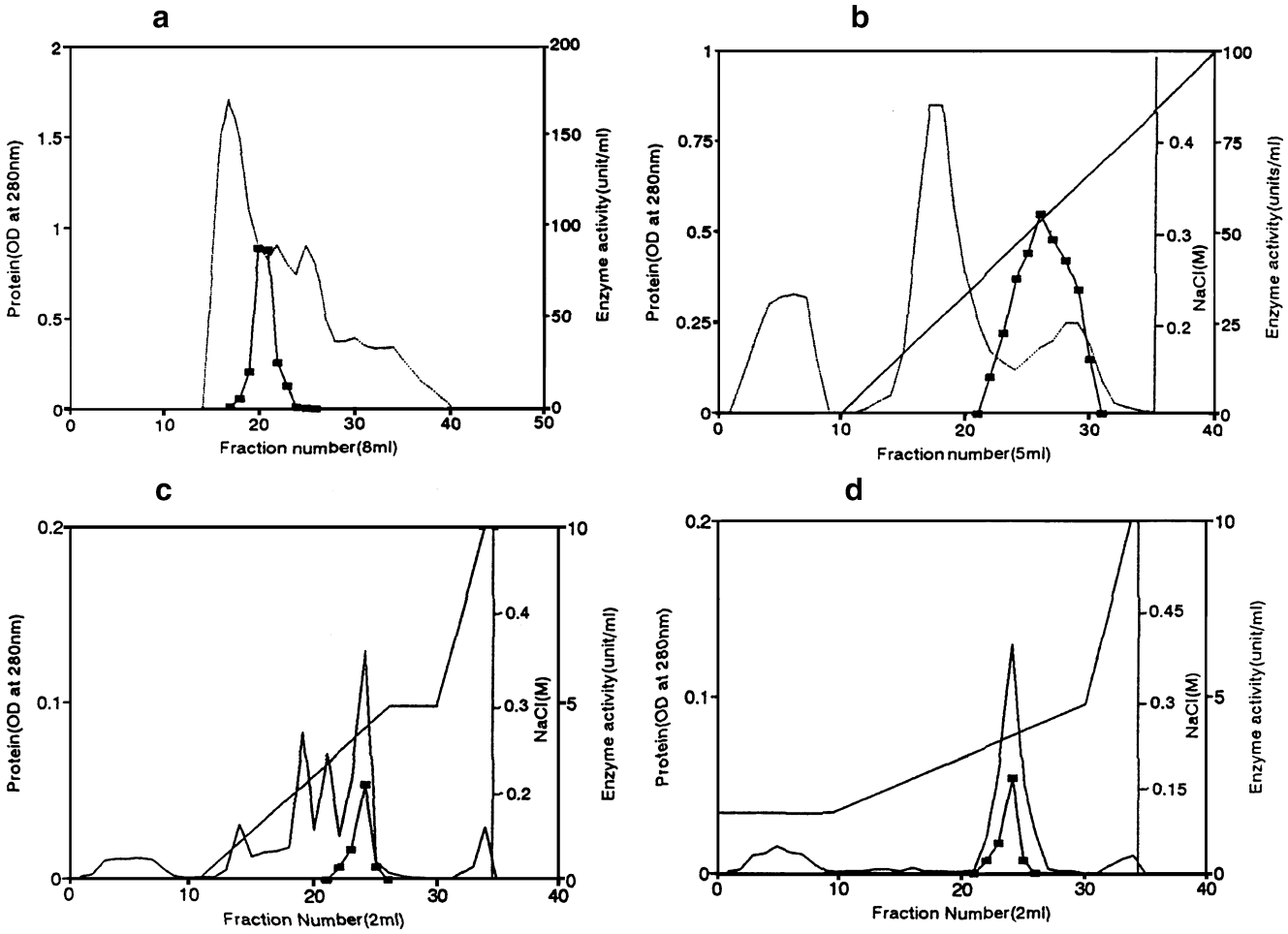

Fig. 2 A representative elution profile of the *T. caldophilus* GK-24 AGPase activity using chromatography. (a) Elution profile of gel filtration on Sephacryl S300. In this and the following figures (b–d) the enzyme activity of the eluted fractions (*filled square*) was assayed, and protein absorbance was monitored at 280 nm (*dotted line*). (b) Elution profile of the AGPase activity after chromatography on DEAE-Sephacel. (c) Elution profile of the AGPase activity after chromatography on first Mono Q. (d) Elution profile of the AGPase activity after chromatography on second Mono-Q

ed, and two corresponding degenerate oligonucleotides were constructed, as described previously [25]. A DNA fragment of 600 bp was obtained using the degenerate primers 1 and 2, and the genomic DNA of *T. caldophilus* GK-24 as the template in a PCR, as the *T. caldophilus* GK-24 AGPase gene sequence is available from GenBank with an accession number AF016923. A 0.6-kb probe was generated by PCR with a digoxigenin labeling kit from Boehringer Mannheim, according to the manufacturer's recommendations. The DNA sequence was determined by the dideoxychain termination method using *Taq* polymerase according to the manufacturer's directions (Perkin–Elmer, USA). To express the enzyme in *E. coli*, the gene containing *Nco*I site was cloned into the pHCE19T(II) expression vector (Takara, Japan). *E. coli* DH5α harboring the pHCE19T(II) expression construct containing AGPase (designated pHGLC) was grown at 37°C in LB broth containing 50 μg/ml ampicillin. After full growth, the cells were harvested and frozen at −70°C until needed. The purification of the AGPase was performed by employing chromatographic methods comprising ion-exchange chromatography and gel permeation chromatography.

Immunoblotting analysis

The purified AGPase (0.3 mg) from *T. caldophilus* GK-24 [1] was dissolved in 0.5 ml of phosphate-buffered saline (PBS). The suspension emulsified by sonication with 0.5 ml of Freund's complete adjuvant (Sigma, USA) was injected subcutaneously into a mouse. The injection procedure was repeated twice at 2-week intervals. The final injection was carried out by adding Freund's incomplete adjuvant to the protein preparations. Antisera were collected 7 days after the final injection. The antibodies were purified by fractionation with sodium sulfate (0–40% saturation) and applied to Western blotting.

Random PCR mutagenesis and screening of mutant-1 and mutant-2 showing UGPase or UNGPase activity

Random mutagenesis of the 874-bp AGPase gene fragment with restriction sites, *Kpn*I (W84) and *Apa*I (R396) was performed by error-prone PCR [30] with minor modifications. The forward primer (primer 1, in Table 1), anneals to the *Kpn*I restriction site (bolded), and the reverse primer (primer 2, in Table 1) harbors an *Apa*I site (bolded) at its 5′ end. Mutagenic PCR mixtures were composed of 20 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM each of dNTPs, 20 ng of pHGLC, 0.5 μM each of primers, and 2.5 U of *Taq* DNA polymerase (Takara). DNA amplification was performed for 30 cycles, each of which consisted of 1 min of denaturation at 94°C, 1 min of primer annealing at 60°C and 2 min of primer extension at 72°C. After error-prone PCR, the randomly mutated *glg*C gene with *Kpn*I-*Apa*I restriction sites was ligated into pHCE19T (II) expression vector. The ligated vector was used for the transformation of *E. coli* DH5α. The glgC mutants were selected by screening the colonies showing UGPase or UNGPase activity. For initial screening of each mutant showing UGPase or UNGPase activity, each recombinant cell extract has been subjected to catalytic activity toward enatiomeric substrates including [glucose-1-phosphate + uridine triphosphate] and [*N*-acetylglucosamine-1-phosphate + UTP] to produce UDP-glucose and UDP-*N*-acetylglucosamine, respectively, by HPLC system (System Gold, Beckman), as described previously [1, 25].

Assays of AGPase, UGPase and UNGPase

Synthesis of sugar-nucleotides (ADP-glucose, UDP-glucose and UDP-*N*-acetylglucosamine) was measured as previously described [1, 25]. The reaction was carried out at 75°C for 10 min in 200 μl of the reaction mixture comprising 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM sugar-1-phosphate, 2 mM ATP or UTP. The reaction was terminated by immersing the Eppendorf tube in ice, and the reaction mixture was analyzed by HPLC system (System Gold, Beckman). A nucleotide analysis column (C-18; 4.6 × 300 mm) was used to resolve NDP-sugar from the substrate NTP and any small amounts of NMP or NDP present. Solvent A was 25 mM K-phosphate buffer (pH 4.0) containing 5 mM tetrabutylammonium chloride and 60% (v/v) acetonitrile. The column was run at a flow rate of 1 ml/min, and the separation was completed in 30 min followed by a 5-min re-equilibration cycle. Detection was performed by measurement of absorption at 254 nm using a Beckman model 166 programmable detector. One unit of sugar-nucleotide pyrophosphorylase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NDP-sugar per minute under the conditions of the assay.

Purifications of mutant-1 and mutant-2 enzymes showing UGPase and UNGPase activities

The cloned mutants-1 and 2 were grown at 37°C in Luria-Broth (LB) medium and were harvested at the logarithmic phase. The frozen cells (5 g) were thawed and resuspended (about 5 ml of buffer per gram of cells) in 50 mM Tris/Cl buffer, pH 8.0 (buffer A) containing 5 mM $MgCl_2$. The cells were disrupted twice by sonic oscillation for 6 min, followed by centrifugation. The supernatant was precipitated between 30 to 70% ammonium sulfate saturation. The precipitates were resuspended in 30 ml of buffer A, dialyzed against 100 vol. of the same buffer, and applied to a gel filtration on Sephacryl S300 (2.1 cm × 78). Then, eluted fractions having enzyme activity were applied to a DEAE-Sephacel column (1.5 × 10 cm) that had been pre-equilibrated with the same buffer. The enzyme was eluted using a linear gradient of 0 to 0.5 M NaCl in 100 ml of buffer A. The pooled active fractions were used for subsequent characterization. For further purification of the enzyme, the active fractions were applied to first Mono-Q and second Mono-Q chromatography with stepwise gradient elution of NaCl concentrations.

Results and discussion

Random PCR mutagenesis using error-prone PCR conditions

The gene for *T. caldophilus* GK-24 AGPase has been cloned into plasmid pHCE19T(II), which was designed to

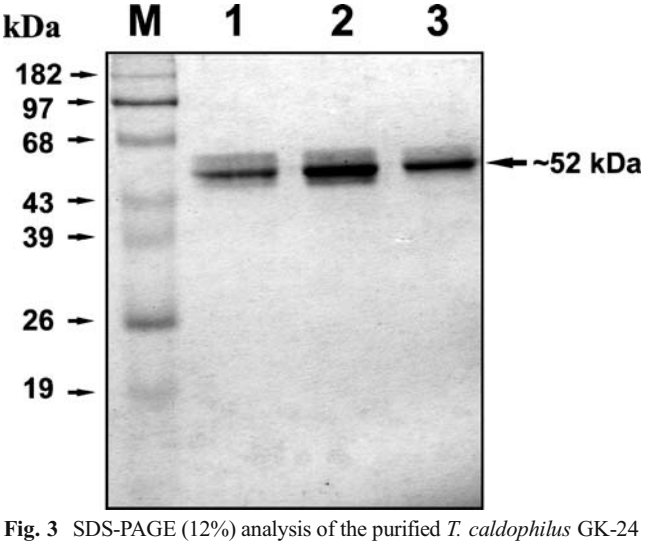

Fig. 3 SDS-PAGE (12%) analysis of the purified *T. caldophilus* GK-24 AGPase. *Lanes1*, purified AGPase (5 μg); *2*, purified mutant-1 enzyme (5 μg); *3*, purified mutant-2 enzyme (5 μg); *M*, standard molecular weight markers containing myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa). *Arrow* indicates the position of the purified AGPase

Table 2 Comparison of the apparent affinities for substrates and specific activities of *T. caldophilus* GK-24 wild-type and mutant enzymes

Clone	Type of pyrophosphorylase	Kinetic parameters				Specific activity[a]		Ref.
		K_m for sugar-1-P (mM)	V_{max} for sugar-1-P (mM/min)	K_m for NTP (mM)	V_{max} for NTP (mM/min)	Units/mg protein	Reaction products	
Wild type	AGPase	4.2 (G-1-P)	32.4 (G-1-P)	1.9 (ATP)	25.7 (ATP)	12.28	ADP-Glc	1
H145G ($n = 2$)	UGPase	0.8 (G-1-P)	8.5 (G-1-P)	2.2 (UTP)	17.0 (UTP)	7.00	UDP-Glc	This study
H145G/A325V ($n = 3$)	UNGPase	1.0 (GlcNAc-1-P)	37.1 (GlcNAc-1-P)	3.0 (UTP)	53.8 (UTP)	10.18	UDP-GlcNAc	This study

The assays were carried out for the enzyme activities with increasing NTP concentrations (0.1, 0.2, 0.5, 1.0 and 5.0 mM) at fixed concentrations of 10 mM sugar-1-P, and also, the increasing sugar-1-P concentration (0.1, 0.2, 0.5, 1.0 and 5.0 mM) at fixed concentrations of 10 mM NTP.
[a] One unit of sugar-nucleotide pyrophosphorylase is defined as the amount of enzyme required to catalyze the formation of 1 μmol of NDP-sugar per minute under the conditions of the assay.

be expressed continuously without any inducer like IPTG in *E. coli* strains, as described previously [25]. Optimal error-prone PCR mutagenesis conditions were determined by amplifying the AGPase gene at five different $MnCl_2$ concentrations. A 0.5 mM $MnCl_2$ concentration was chosen as the optimal error-prone PCR condition, with about 30% of the mutant AGPase clones obtained at this $MnCl_2$ concentration (data not shown). Error-prone PCR mutagenesis of the *glg*C gene, a structural gene for AGPase, was performed to identify the amino acid residues required for AGPase activity. In order to clone a mutant directly into an expression vector (pHGLC), two restriction enzyme sites were selected, as described in Materials and methods. The 874-bp fragment between W84 (*Kpn*I) and R396 (*Apa*I), known as the ATP and G-1-P binding area [31], was randomly mutagenized by error-prone PCR. The forward primer (primer 1) anneals to the *Kpn*I restriction site (bolded), and the reverse primer (primer 2) harbors an *Apa*I site (bolded) at its 5′ end (Table 1). After transformation of *E. coli* DH5α, 656 transformants were pooled into a 96-well plate and were screened for UGPase or UNGPase activity without AGPase activity. Among 656 colonies, two colonies showed UGPase activities and three colonies for UNGPase activities. The plasmid DNA of each clone showing the UGPase or UNGPase activity was sequenced and expressed on large scale for the further purification of the coding enzyme.

Expression of *AGPase*, *UGPase* or *UNGPase* genes in *E. coli* and purification of each enzyme from the mutant

The genes such as *AGPase*, *mutant-1* and *mutant-2*, which were cloned into pHCE19T(II), were expressed in *E. coli* DH5α cells at 37°C. The genes were expressed abundantly in the transformed DH5α cells and enzymes were produced and localized mainly in the cytosolic fraction (Fig. 1), as anti-AGPase polyclonal antibody readily was reacted with the mutant-1 enzyme or mutant-2 enzyme from the recombinant *E. coli* DH5α. This result indicates that the mutant-1 and mutant-2 enzymes are common in their antigenic determinants to the anti-AGPase. For purification of mutant-1 and mutant-2 enzymes from the recombinant strains of *E. coli* DH5α/pHCE-UGP and *E. coli* DH5α/pHCE-UNGP, the cloned mutants were grown at 37°C in LB media and the cell extracts were subjected to 30 to 70% ammonium sulfate saturation. The fractions containing each enzyme from each strain were applied to sequential chromatographic processes such as gel filtration on Sephacryl S300 (2.1 cm × 78), a DEAE-Sephacel column (1.5 × 10 cm), first Mono-Q and second Mono-Q chroma-

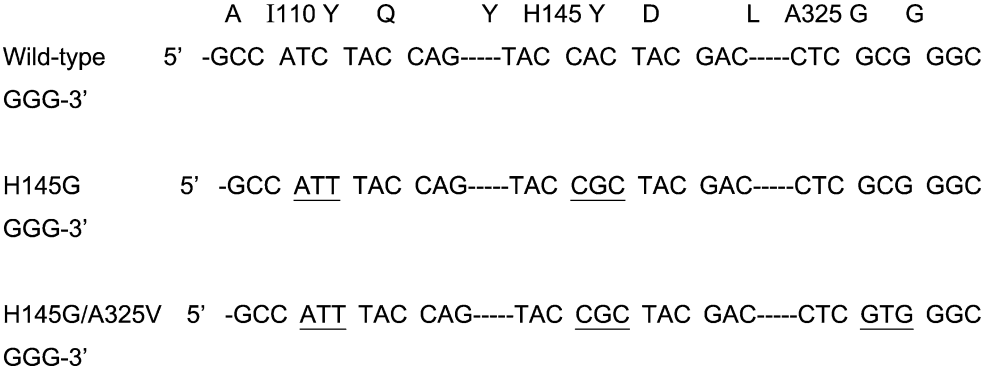

Fig. 4 Nucleotide sequence and encoded protein sequence of *T. caldophilus* GK-24 AGPase and mutants. The position of the mutation, deduced from error-prone PCR and sequencing of mutants having UGPase and UNGPase activities, are shown with the wild-type AGPase

tography, as described in Materials and methods. A representative purification profile of the mutant-1 enzyme from *E. coli* DH5α cells has been shown in Fig. 2. The other two enzymes of AGPase and mutant-2 enzyme have been eluted and purified with the basically same pattern as mutant-1 enzyme (data not shown). The purification procedure resulted in about 55.4 to 66.8-fold purified enzymes with 10–12.5% recoveries for three enzymes. The purified mutant-1 and mutant-2 enzymes are 46-kDa proteins, which have the same molecular weight as the AGPase from *T. caldophilus* GK-24 (Fig. 3).

Kinetic alterations in substrate utilization by error-prone PCR mutants

The apparent kinetic parameters (K_m, V_{max}) for the mutants and their mutated positions on the DNA sequence of the AGPase gene are shown in Table 2 and Fig. 4. I110 remained unchanged because of codon degeneracy, though the DNA sequence (ATC) coding for I110 was changed to ATT. Two mutant clones (H145G), which histidine 145 was substituted by glycine, had an UGPase activity specifically, but not AGPase activity, as the recombinant AGPase enzyme has no residual activity for UTP and/or GlcNac-1-P. The specific activity of the H145G mutant enzyme (7.00 U/mg protein for UGPase) was much lower than that of AGPase of the wild type (12.28 U/mg protein) (Table 2). The mutant-1 enzyme has no any residual UNGPase activity. On the other hand, three mutants (H145G/A325V), having the double mutations at histidine residue 145 to glycine residue and alanine residue 325 to valine residue, showed an UNGPase, but not AGPase activity. The H145G/A325V mutant had a slightly lower specific activity (10.18 U/mg protein for mutant-2 enzyme) than that of the wild type AGPase (12.28 U/mg protein).

The position of G145 residue of the mutant showing UGPase activity is located in the vicinity of Asp142, a residue that has been reported to be very important for AGPase catalytic activity in *E. coli* [31]. Moreover, H145 of *T. caldophilus* AGPase was located near the ATP binding region, which also contains Y114, a residue known to be involved in ATP binding in *E. coli*. These results suggest that H145 of *T. caldophilus* AGPase is involved in the binding of ATP and, if it is changed to glycine, the specificity for base alters to UTP. Some alterations in the specificity were observed in the mutant H145G/A325V, as are seen in Table 2. When A325 is replaced by valine, the mutant showed an UNGPase activity. This result indicates that A325 is associated with sugar binding and confers the specificity for the sugar substrates and V325 of the mutant appears to be indirectly involved in the binding of the *N*-acetylamino group of *N*-acetylglucosmine-1-phosphate (GlcNAc-1-P). The position of sugar binding in AGPase of *T. caldophilus* is somewhat controversial, considering the previous report that G335 is the amino acids involved in the enzyme regulation, not sugar binding, in *E. coli*.

The three-dimensional structure of AGPase would be helpful to answer for structure-function relationship. Recently, Jin *et al*. [32] reported crystal structure of potato tuber ADP-glucose pyrophosphorylase, although it is not a bacterial source. Although it is not a bacterial structure, the active site (and hence the binding of substrates) may be similar to those of the bacterial enzymes. Structural basis of the AGPase, mutant-1 and mutant-2 enzymes will allow fine positions for substrate recognition. Therefore, the existing questions might be clearly answered using more crystal structures of AGPases.

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