Purification and characterization of a thermostable ADP-glucose pyrophosphorylase from *Thermus caldophilus* GK-24

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An extremely thermostable ADP-glucose pyrophosphorylase (AGPase) has been purified from *Thermus caldophilus* GK-24 to homogeneity by chromatographic methods, including gel filtration and ion-exchange and affinity chromatography. The specific activity of the enzyme was enriched 134.8-fold with a recovery of 10.5%. The purified enzyme was a single band by SDS/PAGE with a molecular mass of 52 kDa. The homotetrameric structure of the native enzyme was determined by gel filtration analysis, which showed a molecular mass of 230 kDa on a Superose-12 column, indicating that the structure of the enzyme is different from the heterotetrameric structures of higher-plant AGPases. The enzyme was most active at pH 6.0. The activity was maximal at 73–78 °C and its half-life was 30 min at 95 °C. Kinetic and regulatory properties were characterized. It was found that

AGPase activity could be stimulated by a number of glycolytic intermediates. Fructose 6-phosphate, fructose 1,6-bisphosphate, phenylglyoxal and glucose 6-phosphate were effective activators, of which fructose 1,6-bisphosphate was the most effective. The enzyme was inhibited by phosphate, AMP or ADP. ATP and glucose 1-phosphate gave hyperbolic-shaped rate-concentration curves in the presence or absence of activator. A remarkable aspect of the amino acid composition was the existence of the hydrophobic and Ala + Gly residues. The N-terminal and internal peptide sequences were determined and compared with known sequences of various sources. It was apparently similar to those of AGPases from other bacterial and plant sources, suggesting that the enzymes are structurally related.

INTRODUCTION

ADP-glucose pyrophosphorylase (ATP:α-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27; AGPase) is one of the major regulatory enzymes in the biosynthesis of α-glucans in bacteria and plants for generating the glucosyl donor ADP-glucose [1–3]. This enzyme catalyses the following reversible reaction:

Its regulatory and catalytic properties from several sources have been exclusively reviewed by Preiss et al. [4]. It has been generally accepted that AGPase is commonly modulated by allosteric effectors and is a tetrameric protein [3]. However, major differences in allosteric properties and protein structure exist between the higher-plant and bacterial enzymes [1,2,4]. The most notable characteristics are that 3-phosphoglycerate (3PGA) and P_i are the positive and negative effectors respectively of all the plant AGPases so far studied [4]. The regulatory effectors of enteric and anaerobic photosynthetic bacterial enzymes are different, being fructose 1,6-bisphosphate (F1,6P_2), fructose 6-phosphate (F6P) or pyruvate, depending on the system studied [3,5]. The molecular mass of AGPase from different sources, both bacteria and plants, has been determined as approx. 200 kDa, with four subunits. In enteric bacteria, AGPase is a homotetramer encoded by a single gene locus [3], whereas the higher-plant enzymes are more complex, having a heterotetrameric structure with two dissimilar subunits [6–12].

So far, bacterial AGPases have been purified and extensively characterized from cyanobacteria, *Escherichia coli* and *Salmon-*

ella typhimurium. Cyanobacteria are considered to be phylogenetic intermediates between plants and bacteria. The cyanobacterial AGPases from *Synechocystis* PCC6803 and *Anabaena* PCC7120 were purified and characterized previously [13–15]. As expected, the cyanobacterial enzymes have characteristics intermediate between those of the higher-plant and *E. coli* enzymes. Sequencing of genomic molecular clones of AGPase isolated from *Synechocystis* PCC6803 and *Anabaena* PCC7120 show that the cyanobacterial enzymes have a greater similarity to the higher-plant enzyme than to the bacterial enzymes [14,15].

Because the bacterial and plant AGPases catalyse the same reaction, their structural dissimilarities may reflect their different specificities for allosteric regulators. A comparison of the regulatory and structural properties of AGPase from bacteria and higher plants might suggest a possible relationship, i.e. the specificity for 3PGA and P_i as regulators might require an enzyme with a heterotetrameric structure. The cyanobacterial AGPases from *Synechocystis* PCC6803 and *Anabaena* PCC7120 were found to be specifically regulated by 3PGA and P_i . Furthermore they are antigenically more related to the higher-plant enzyme than to the bacterial enzymes, which are homotetrameric [13]. In *E. coli* and *S. typhimurium*, the enzyme was also elucidated as being homotetrameric in structure and allosterically activated by F1,6P_2 , pyridoxal 5'-phosphate (PLP), NADPH and 1,6-hexanediol, and inhibited by 5'-AMP, ADP and P_i [16,17].

Extremely thermophilic bacteria belonging to the genus *Thermus* can routinely grow at temperatures of 70–75 °C, and some species can grow at 85 °C [18]. Various highly thermostable enzymes, which have been reported to be useful in high-temperature

Abbreviations used: AGPase, ADP-glucose pyrophosphorylase; F1,6P_2 , fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; 3PGA, 3-phosphoglycerate; PLP, pyridoxal 5'-phosphate.

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systems, have been isolated from thermophilic bacteria [19–22]. In particular, carbohydrate-related enzymes from extreme thermophiles have attracted interest because of their application to biomass bioconversions. However, there has been no report on the formation and biochemical characteristics of thermophilic enzymes used in carbohydrate biosynthesis from *Thermus* species. Moreover, AGPase from *Thermus* species has not yet been studied. A comparison of the properties and primary structures of the thermostable AGPase with those of the enzyme from other sources will give insight into structure and function of this protein.

In the course of study on the strain, it was shown that the organism synthesizes glycogen as the major carbohydrate reserve (J. H. Ko, C.-H. Kim, D.-S. Lee and Y. S. Kim, unpublished work), in a similar manner to that observed in bacteria. The evolutionary relationships of *Thermus* to prokaryotes and plant prompts a comparison of *Thermus* AGPase properties with those of *E. coli*, *S. typhimurium*, cyanobacteria and plants. In the present study we have purified the AGPase from *T. caldophilus* GK-24 and compared the molecular mass, N-terminal amino acid sequence, internal amino acid sequence and heat stability of the enzyme from *T. caldophilus* GK-24 with the enzymes from other bacteria [14–17] and plants [4–12].

MATERIALS AND METHODS

Chemicals

Standard ADP-glucose, ATP, ADP, AMP, F6P, F1,6P₂ and 3PGA were purchased from Sigma Co. DEAE-Sephacel, Affi-Gel Blue and Mono-Q columns were products of Pharmacia Co. All the reagents were of the highest grade available.

Micro-organism and cultivation

T. caldophilus GK-24 cells (kindly supplied by Professor T. Ohta, Faculty of Agriculture, The University of Tokyo, Tokyo, Japan) [19,20] were grown in a medium containing 0.8% polypeptone, 0.4% yeast extract and basal salts with 1% (w/v) glucose as a carbon source [21]. The pH of the medium was adjusted to 7.2. The cultivation was performed at 75 °C for 24 h in a jar fermenter in 10 litres of medium with an inoculum of 500 ml of culture. The previous seed culture medium was made of 0.4% polypeptone, 0.2% yeast extract and basal salts.

Assay of AGPase

Synthesis of ADP-glucose was measured with HPLC by a slight modification of that described in [23]. The reaction was conducted at 75 °C with 200 µl of the reaction mixture (20 mM Tris/HCl (pH 7.6)/5 mM MgCl₂/5 mM glucose 1-phosphate/2 mM ATP) for 5 min. After terminating the reaction by immersing the Eppendorf tube in ice, the reaction mixture was subjected to analysis by HPLC. One unit of AGPase activity is defined as the amount of enzyme that catalyses the formation of 1 µmol of ADP-glucose per min at 75 °C.

The HPLC analysis was performed with a System Gold, consisting of a model 126 programmable solvent module, a model 166 programmable detector module and Premium 286 personal computer. An ODS C18 (5 µm) reverse-phase column (4.6 mm × 300 mm), with tetrabutylammonium chloride as ion-pair reagent, was used in various separation modes. Standards were prepared at concentrations of approx. 1–2 mM. The injection volumes of both the standard mixture and the sample were 50 µl. Peaks were identified from their retention times and absorbance at 254 nm. The mobile phase was 25 mM phosphate,

pH 4.0, containing 5 mM tetrabutylammonium chloride and 60% (v/v) acetonitrile gradient.

Purification of the thermostable AGPase from *T. caldophilus* GK24

Preparation of crude extract and ammonium sulphate fractionation

Unless otherwise stated, all steps were performed at 4 °C. The cells were grown at 75 °C in the medium as described [21] and harvested at the logarithmic phase (approx. 2×10^9 cells/ml). The frozen cells (15 g) of *T. caldophilus* GK24 were thawed and resuspended (about 5 ml of buffer per g of cells) in 50 mM Tris/Cl buffer, pH 8.0 (buffer A), containing 5 mM MgCl₂. The cells were disrupted twice by sonic oscillation in a Ultrasonic homogenizer (model Cole-Parmer 4710 series) for 6 min and followed by centrifugation at 12000 g for 20 min. The supernatants are referred to as the crude extract. The crude extract fraction was brought to 30% saturation with crystalline ammonium sulphate and then centrifuged at 12000 g for 15 min. The pellet was discarded and the supernatant brought to 70% saturation with ammonium sulphate. This second precipitate was collected by centrifugation at 12000 g for 20 min, dissolved in a small volume of buffer A, and dialysed against 100 vol. of the same buffer.

Sephacryl-S200 gel filtration chromatography

The dialysed sample (60 ml) was divided into 10 fractions and applied to a Sephacryl-S200 column (2.5 cm × 120 cm) that had been equilibrated with buffer A. Fractions of 8 ml each were collected and enzyme activity was detected between fractions 17 and 24 (results not shown). The active fractions were pooled and then concentrated 10-fold by ultrafiltration with a Diafuro YM 30 membrane (Amicon Co., Beverly, MA, U.S.A.).

DEAE-Sephacel chromatography

The concentrated enzyme solution (24 ml) was further dialysed against buffer A and applied to a DEAE-Sephacel column (1.5 cm × 20 cm) that had been pre-equilibrated with the same buffer. After washing with 1.5 litres of buffer A, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in 300 ml of buffer A. Enzyme activity was detected in the region of 0.25–0.34 M NaCl and the active fractions (5 ml per fraction, nos. 21 to 30) were pooled (50 ml) and then concentrated in the manner indicated above (results not shown).

Affi-Gel Blue affinity chromatography

The sample was diluted 3-fold in buffer A and applied to an Affi-Gel Blue column (1.2 cm × 12 cm), which was manually packed into an HR10/10 column equilibrated with buffer A. After being washed with 2 litres of buffer A, the enzyme was eluted with a step gradient (five bed volumes) of buffer A containing 10 mM ATP. Active fractions were collected (40 ml), dialysed against buffer A and concentrated by the method described above (results not shown). The purified enzyme was stored at –20 °C in a 50 µg/ml aqueous solution.

First and second Mono-Q chromatography

Further purification of the AGPase enzyme protein was achieved by high-performance ion-exchange chromatography on a Mono-Q HR 10/10 column (10 mm × 100 mm) (the first Mono-Q). The concentrated sample (5 ml) was applied to the column pre-equilibrated with buffer A. The column was washed with five column volumes of the same buffer and the enzyme activity was eluted with a linear NaCl gradient (100 ml, 0–0.5 M)

in buffer A at a flow rate of 0.5 ml/min. Enzyme activity was detected in the region of 0.24–0.28 M NaCl concentration; the active fractions of 6 ml were collected and those containing activity were pooled (results not shown). The collected fractions were re-applied to the same column (the second Mono-Q) and the AGPase was recovered as a single peak (results not shown) that eluted at 0.25–0.27 M NaCl, where the enzyme activity was found to coincide with the protein peak. At this stage of purification, the AGPase was found to be free from contaminating proteins and the purified AGPase was used for the next characterization.

SDS/PAGE and native PAGE

Protein extracts at different stages of purification were analysed by PAGE [12 % (w/v) acrylamide] under denaturing conditions by the method of Laemmli [24]. The molecular mass of the polypeptide present in the purified enzyme fraction was determined from a graph of migration distance against the logarithm of the molecular mass of standard polypeptides. Alternatively, the proteins were subjected to electrophoresis on 12 % polyacrylamide gel with the PhastSystem (Pharmacia) in accordance with the manufacturer's instructions. The proteins were revealed by staining with Coomassie Blue or silver. Native PAGE was performed by the method of Davis as described by Weber and Osborn [25].

Determination of molecular mass

The molecular mass of the purified enzyme was estimated by SDS/PAGE and gel filtration. SDS/PAGE was performed with the following markers: myosin, 200 kDa; phosphorylase b, 97.4 kDa; BSA, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β -lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa. The molecular mass of the native AGPase was estimated by gel filtration on a column of Superose-12 HR 10/30 with an FPLC system. The column was equilibrated in 50 mM Tris/Cl, pH 7.6, containing 100 mM NaCl and 10 mM 2-mercaptoethanol as the equilibration/elution buffer. Thyroglobulin (669 kDa), apoferrin (443 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase were used as standard proteins. The void volume was measured by the elution of a sample of Blue Dextran 2000. The reference and sample proteins were applied individually in a volume of 0.5 ml with the manual V-7 injector of the Pharmacia FPLC system. The FPLC system was kept at a constant flow rate of 0.4 ml/min at room temperature during elution. The apparent molecular mass of the enzyme was estimated from a plot of V_e/V_0 (elution volume) against the logarithm of the molecular mass of the standard proteins.

N-terminal and internal amino-acid sequencing, and amino acid composition of AGPase

The N-terminus of AGPase was sequenced with a gas-phase peptide sequencer (Applied Biosystems model 477A) equipped with PTH-Xaa analyser (Applied Biosystems model 120A). Phenylthiohydantoin derivatives of amino acids (PTH-Xaa) were identified in accordance with the manufacturer's recommended procedures. To determine the internal sequence, the AGPase protein was digested with endopeptidase Glu-C (Wako Pure Chemicals, Osaka, Japan). The resulting peptides were separated by HPLC on an ODS C-8 column (2.1 mm $\times$ 150 mm) and eluted with a linear gradient of 0–70 % (v/v) acetonitrile in trifluoroacetic acid at a flow rate of 150 μ l/min. The eluted peptides were detected at 210, 277 and 292 nm. Peptide sequences were determined by a gas-phase peptide sequencer as described above.

Amino acids were identified and measured with a Hitachi 833 amino acid analyser after an enzyme preparation had been hydrolysed in 6 M HCl *in vacuo* for 24 h at 110 °C, as described by Kim and Kim [26]. Briefly, the enzyme sample was thoroughly washed with distilled water by using a Centricon YM10 (Amicon) microconcentrator. The sample (50 μ l, 42 μ g) protein was freeze-dried, mixed with 30 μ l of 4.2 M NaOH and heated at 120 °C for 24 h *in vacuo* in a sealed tube. The hydrolysate was neutralized with 6 M HCl, made up to 30 μ l with distilled water, and analysed for tryptophan with the amino acid analyser. An enzyme sample (30 μ l) was mixed with 30 μ l of 12 M HCl and 60 μ l of 6 M HCl, heated for 24 h and evaporated *in vacuo*. The residue was dissolved in 20 μ l of 0.1 M sodium phosphate, pH 6.8, kept at 25 °C for 4 h to oxidize cysteine to cystine, and mixed with 80 μ l of 0.2 M sodium citrate, pH 2.2. After being passed through a disc membrane filter, the solution was tested with the amino-acid analyser.

Modification with PLP

Purified enzyme (0.1 mg) was modified with various amounts of PLP (10, 20, 50 and 100 μ mol) in 100 μ l mixtures containing 50 mM Tris/Cl, pH 8.0. The reactions, which were conducted at room temperature, were started by the addition of PLP; every 5 min an aliquot (10 μ l) was assayed at 75 °C. Another aliquot was reduced by the addition of sodium borohydride at 50 mM. As a control, distilled water instead of PLP was used. Where PLP and sodium borohydride were included during modification, the reaction mixture was dialysed against buffer A.

Analytical methods

Protein concentration was determined by using Bradford reagent [15] with BSA as standard.

RESULTS AND DISCUSSION

Purification of thermostable AGPase from *T. caldophilus* GK24

T. caldophilus GK24 AGPase was purified to homogeneity by sequential chromatographic methods including Sephacryl S-200, DEAE-Sephacel, Affi-Gel Blue affinity and Mono-Q chromatography. Table 1 summarizes a typical purification of AGPase from 15 g of *T. caldophilus* GK-24 cells. The purification procedure resulted in a 134.8-fold purified enzyme with a specific activity of 161.7 units/mg of protein and 10.5 % recovery. Of interest is that Affi-Gel Blue was particularly effective in purification of the enzyme. The specific activity of the purified enzyme is similar to the values reported for AGPases from other bacteria (114 units/mg) [27], cyanobacteria (133.9 units/mg) [13] and higher plants (120 units/mg) [9].

Purity and molecular mass of the purified enzyme

After SDS/PAGE of the final enzyme purification, staining the polyacrylamide gel with Coomassie Blue yielded a single band, indicating a purity higher than 95 % by this criterion (Figure 1). The single band indicated a molecular mass of 52 kDa. In contrast, molecular mass determination of the native enzyme with gel-filtration chromatography on Superose-12 gel equipped with the FPLC system (Pharmacia Co.), revealed a molecular mass (average of three separate determinations) of 230 ± 5 kDa (results not shown). This result indicates that the native enzyme has a homotetrameric structure, consisting of four subunits of equal size. This was also confirmed by N-terminal sequencing, as described below. Although it has been suggested that AGPases

Table 1 Summary of purification of AGPase from *T. caldophilus* GK24

The results correspond to a typical purification from 15 g of wet cells. One unit of AGPase activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol of ADP-glucose per min at 75 °C.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold purification
Crude enzyme	650.0	783.4	1.2	100	1
(NH ₄) ₂ SO ₄ precipitate (30–70%)	615.0	803.3	1.3	102.5	1.1
Sephacryl-S200	112.0	562.5	5.0	71.8	4.2
DEAE-Sephacel	18.0	482.7	26.8	61.6	22.3
Affi-Gel Blue	3.0	364.2	121.4	46.5	101.2
Mono-Q (first)	1.2	163.0	135.8	20.8	113.2
Mono-Q (second)	0.51	82.5	161.7	10.5	134.8

Figure 1 SDS/PAGE [12% (w/v) gel] analysis of the purified *T. caldophilus* GK-24 AGPase

Lane 1, purified AGPase (1 μ g); lane 2, purified AGPase (3 μ g); lane 3, standard molecular mass markers containing myosin (200 kDa), phosphorylase *b* (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). The arrow at the left indicates the position of the purified AGPase.

from many plants and *Chlamydomonas reinhardtii* [28] are composed of two different subunits with molecular masses of 50 and 53 kDa [4], the AGPases from bacterial sources are composed of the same subunits, as elucidated from *E. coli* [3,17,29] and other cyanobacteria [13–15]. It is therefore likely that the *T. caldophilus* GK24 AGPase has the same molecular properties as those of other bacteria.

Effects of temperature and pH on enzyme activity

T. caldophilus GK-24, an extremely thermophilic bacterium, was grown at 73–78 °C. Its AGPase, which had the highest activity at 73 °C, possessed high thermostability, the half-life of purified AGPase being 30 min at 95 °C (results not shown). The pH optimum for the enzyme activity was rather broad, being between pH 6 and 7. This was independent of the buffer used in the reaction mixtures as well as the assay conditions. The enzymic activity was further enhanced by F6P, F1,6P₂ and G6P in the pH range 6–8 (results not shown).

Requirements for enzyme activity

AGPase activity was dependent on the simultaneous presence of Mg²⁺ ions, BSA, PP_i, ADP-glucose and enzyme (results not

Figure 2 Kinetic parameters of the purified *T. caldophilus* GK-24 AGPase *v* plotted against *v*/[*S*] with the Woolf and Hofstee equation

(a) The effect of ATP concentration on the activity of purified AGPase with increasing ATP concentration (0.1, 0.2, 0.5, 1.0 and 5 mM) at a fixed concentration of 10 mM G1P. (b) The effect of G1P concentration on the activity of purified AGPase with increasing G1P concentration (0.1, 0.2, 0.5, 1.0 and 5 mM) at a fixed concentration of 10 mM ATP.

shown). Mg²⁺ was required for enzyme activity. Although Co²⁺ and Mn²⁺ were found to be able to replace Mg²⁺ in enzyme reactions, they were not as effective as Mg²⁺ (results not shown). These results are similar to those for *E. coli* AGPase [30]. BSA usually stimulated the enzyme activity by 30–40 %. Sugar nucleotide synthesis was not observed when UTP, GTP, ITP, CTP or dATP was used in place of ATP.

With increasing concentration of ATP or glucose 1-phosphate (G1P) as substrate in the reaction solution containing 0.1 μ g of the purified enzyme, the rate of ADP-glucose synthesis increased and a typical Michaelis–Menten substrate saturation pattern was obtained. Furthermore both ATP and G1P gave a hyperbolic-shaped rate–concentration curve in the presence or absence of activator. From Woolf and Hofstee plots, apparent *K_m* and *V_{max}* values for ATP were 1.9 mM and 25.7 respectively (Figure 2a), and those for G1P were 4.2 mM and 32.4 units/min (Figure 2b)

Table 2 Effect of different metabolites on the activity of AGPase purified from *T. caldophilus* GK-24

AGPase activity was assayed in 200 μ l of reaction mixture containing 0.1 μ g of enzyme and 1 mM metabolites.

Compound	$10^{-3} \times$ AGPase activity (units)	Relative activity
None	22.3	1.0
ADP	19.5	0.9
AMP	13.9	0.6
F6P	40.0	1.8
F1,6P ₂	44.8	2.0
G6P	34.0	1.5
Pyruvate	22.3	1.0
3PGA	22.4	1.0
PP _i	13.8	0.6
P _i	13.9	0.6
PLP*	9.6	0.4
Phenylglyoxal	25.2	1.1

* The enzyme solution, after preincubation for 30 min with 1 mM PLP, was reduced with NaBH₄ and then assayed for activity.

respectively. These results are similar to those for other bacteria and plants [6–12,13–15,30].

Effects of various metabolites on enzyme activity

To evaluate the possible regulation of thermophilic AGPase *in vivo*, the effects of different metabolites on the physiological activity of the purified AGPase were studied, as shown in Table 2. ADP, AMP and P_i are made by AGPase as products from ATP and G1P. At 1 mM concentration, enzyme activity was inhibited by 10–40 %. These levels of inhibition have been reported for the enzyme from many bacteria [3] and plants [4,5]. Other compounds were added to elucidate the possible physiological regulation of AGPase from *T. caldophilus* GK-24 and to compare the results with those previously reported for many bacteria [3]. F1,6P₂, F6P, 3PGA and pyruvate are known as the most prevalent activators. F6P (1.8-fold) and G6P (1.5-fold) activated the *T. caldophilus* GK24 AGPase, but not as well as F1,6P₂, which was the most effective activator of all glycolytic intermediates tested. The activation by F1,6P₂ of the AGPase under reaction conditions was approx. 2.0-fold (Table 2). In contrast, even though 3PGA is generally known as the most prominent allosteric activator in various plants [4,5], the activation by 3PGA was minimal, and far less than G6P, F6P or F1,6P₂, indicating that AGPase from *T. caldophilus* is quite different from AGPases from other sources.

Furthermore even though PLP, known as a lysine-modifying reagent, has been noted as an allosteric activator and its interaction with the allosteric site of AGPase has been extensively characterized in *E. coli* [17,31,32], this reagent decreased the enzymic activity to 60 %, whenever PLP itself or the reduced enzyme–PLP complex form were used (results not shown). Seemingly, although phenylglyoxal, a known arginine-modifying reagent, was found to be an inhibitor of AGPase in *E. coli* and spinach [33], it activated the enzyme in *T. caldophilus* GK-24 (Table 2). This suggests that a lysine residue might not be located at the allosteric activator site, or has been changed to an arginine in thermophilic AGPase. These facts have also been mentioned for *T. aquaticus* YT-1 DNA polymerase [34]: when the amino acid composition of *T. aquaticus* DNA polymerase I was compared with that of *E. coli* DNA polymerase I, it was

Bacterial strains	Amino-terminal sequence
I	Met-Val-(Lys)-Val-Glu-Val-Leu-Gly-Met-iLe-Leu-Ala-Gly-Gly-Gln-Gly-Ser-Leu-Tyr-Ser-Leu-Thr-Ala
II	Met-Val-Ser-Leu-Glu-Lys-Asn-Asp-His-Leu-Val-Leu-Ala-Arg-Gln-Leu-Pro-Phe-Thr-Leu-Val
III	Met-Val-Ser-Leu-Glu-Lys-Asn-Asp-His-Leu-Val-Leu-Ala-Arg-Gln-Leu-Pro-Phe-Thr-Leu-Val
IV	Met-Lys-Ala-Gln-Pro-Xaa-Leu-Arg-Thr-Ala-Gln-Ala-Met-Phe-Val-Arg-Gln-Leu-Gln-Lys-Ser-Val
V	Met-Lys-Gln-Ile-Thr-Glu-Thr-Gln-Met-Asp-Ile-Asn-Arg-Leu-Thr-Glu-Xaa-Xaa-Ala ²⁰
VI	Met-Lys-Lys-Val-Leu-Ala-Ile-Ile-Glu-Gly-Gly-Glu-Gly-Thr-Leu-Tyr-Pro-Thr-Lys-Leu-Arg-Val

Figure 3 N-terminal amino acid sequence comparison of AGPases from various bacterial sources

N-terminal sequences of AGPases from (I) *T. caldophilus* GK24 (this study), (II) *E. coli* [33,34], (III) *S. typhimurium* [35], (IV) *R. sphaeroides* [36], (V) *R. rubrum* [37] and (VI) *Anabaena* sp. [38] are aligned. Identical amino acid sequences between *T. caldophilus* GK24 AGPase and the others are indicated in reversed type.

a	271	Ala-Ile-Asp-Asp-Pro-Ala-Lys-	Tyr-Pro-Tyr-Ile-Ala-Ser-Met-Gly-Val	287
b	238	Leu-Asp-Asp-Ala-Arg-Ala-Lys-Glu-Met-Pro-Tyr-Ile-Ala-Ser-Met-Gly-Ile		255
c	309	Tyr-Ala-Ile-Asp-Asp-Ala-Gln-Lys-Tyr-Pro-Tyr-Leu-Ala-Ser-Met-Gly-Ile		326
d	286	Leu-Asp-Asp-Lys-Arg-Ala-Lys-Glu-Met-Pro-Phe-Ile-Ala-Ser-Met-Gly-Ile		303
e	208	Leu-Asp-Asp-Lys-Arg-Ala-Lys-Glu-Met-Pro-Phe-Ile-Ala-Ser-Met-Gly-Ile		225
f	244	Leu-Ser-Ala-Glu-Lys-Ala-Lys-Leu-Asn-Pro-Tyr-Ile-Ala-Ser-Met-Gly-Ile		261
g	196	Leu-Thr-Pro-Glu-Gln-Ala-Ala-Ser-Gln-Pro-Tyr-Ile-Ala-Ser-Met-Gly-Ile		213
h	196	Leu-Ser-Ala-Glu-Lys-Ala-Lys-Leu-Asn-Pro-Tyr-Ile-Ala-Ser-Met-Gly-Ile		213
i	202	Met-Pro-Asn-Asp-Pro-Ser-Lys-	Ser-Leu-Ala-Ser-Met-Gly-Ile	215
j	202	Met-Leu-Gly-Asp-Ala-Ser-Lys-	Ser-Leu-Ala-Ser-Met-Gly-Ile	215
k		Pro-Lys-Pro-Ile-Pro-Gly-Arg-Pro-Asp-Met-Ala-Leu-Ala-Ser-Met-Gly-Ile		

Figure 4 Comparison of the partial amino acid sequences of *T. caldophilus* GK-24 AGPase with the amino acid sequences of nine other AGPases

Sequences from (a) wheat large subunit [42], (b) wheat small subunit [43], (c) maize [44], (d) potato large subunit [40], (e) potato small subunit [39], (f) rice [41], (g) *Anabaena* [14], (h) *Synechocystis* PCC 6803 [15], (i) *E. coli* [33,34], (j) *S. typhimurium* [35] and (k) *T. caldophilus* GK24 (this study) are aligned. Identical amino acid sequences between thermostable AGPase and other AGPases are indicated in reversed type.

particularly notable that the Arg-to-Lys ratio for the *T. aquaticus* enzyme was nearly twice that of the enzyme from *E. coli*. It was suggested that the propensity of thermophilic proteins to contain Arg rather than Lys residues is simply a reflection of the high GC content of thermophilic organisms [34]. Therefore when the *T. caldophilus* AGPase gene has been cloned and sequenced, a comparison of the activator site of the AGPase with those of other sources will provide genetic information about the evolution of *Thermus* genes.

Comparison of N-terminal and internal amino-acid sequences

The N-terminal amino acid sequence of the AGPase was determined for 23 residues by the Edman degradation method (Figure 3). The sequence was Met-Val-(Lys)-Val-Glu-Val-Leu-Gly-Met-iLe-Leu-Ala-Gly-Gly-Gln-Gly-Ser-Leu-Tyr-Ser-Leu-Thr-Ala. It was compared with those of AGPases from *E. coli* [35,36], *S. typhimurium* [37], *Rhodospseudomonas sphaeroides* [38], *Rhodospirillum rubrum* [39] and *Anabaena* sp. [40]. This sequence shows some similarity to AGPases from the enzyme sources described above (Figure 3): Met¹-Val², Glu⁵, Leu⁷, Met⁹, Leu¹¹-Ala¹², Leu¹⁹, Ser²¹ and Ala²⁴ coincide with the sequences from the similar, noted AGPases. Among them, Leu⁷ and Ala¹² were highly conserved in all the sequences.

In addition, one peptide obtained by endopeptidase Glu-C digestion was sequenced. The obtained sequence (Pro-Lys-Pro-Ile-Pro-Gly-Arg-Pro-Asp-Met-Ala-Leu-Ala-Ser-Met-Gly-Ile) was quite similar to the known sequence of various sources described above (Figure 4).

Amino-acid composition of *T. caldophilus* GK24 AGPase

The amino acid composition of AGPase is presented in Table 3. A remarkable aspect of the composition is the existence of the hydrophobic residues leucine, valine and proline. On the basis of

Table 3 Amino acid composition of AGPases from *T. caldophilus* GK24 (this study), *Anabaena*, *E. coli*, potato small subunit, potato large subunit and rice

Amino acid	<i>T. caldophilus</i> GK24*		<i>Anabaena</i> ‡		<i>E. coli</i> §		Potato small subunit		Potato large subunit¶		Rice**	
	Mol%	Residues†	Mol%	Residues	Mol%	Residues	Mol%	Residues	Mol%	Residues	Mol%	Residues
Asp			7.2	31	6.7	29	7.2	32	6.9	36	6.6	32
Asn			4.0	17	4.4	19	4.3	19	5.0	26	5.8	28
Asx	3.1	13	11.2	48	11.1	48	11.5	41	11.9	62	12.4	60
Glu			5.8	25	6.7	29	5.4	24	4.8	25	6.4	31
Gln			4.0	17	3.0	13	2.9	13	2.9	15	3.3	16
Glx	10.4	36	9.8	42	9.7	42	8.1	37	7.7	40	9.7	47
Thr	3.3	15	5.1	22	2.8	12	4.3	19	3.8	20	3.7	18
Ser	3.6	18	5.1	22	7.0	30	5.9	26	8.6	45	6.0	28
Gly	8.4	59	6.5	28	6.3	27	7.9	35	7.3	38	7.7	37
Ala	11.5	67	7.5	32	7.2	31	9.0	40	8.8	46	8.5	41
Cys	0.8	4	1.4	6	2.1	9	1.4	6	1.3	7	1.0	5
Val	9.6	43	6.3	27	8.6	37	5.9	26	6.1	31	6.8	33
Trp	1.5	4	0.9	4	1.4	6	0.7	3	0.6	3	0.6	3
Met	4.0	14	2.1	9	3.2	14	2.5	11	2.7	14	2.5	12
Ile	4.4	18	10.3	44	5.8	25	9.3	41	8.4	44	8.7	42
Leu	6.4	25	8.2	35	8.6	37	8.4	37	8.1	42	8.1	39
Tyr	4.3	13	4.0	17	1.4	6	4.5	20	3.8	20	4.1	20
Phe	3.2	10	4.2	18	3.5	15	3.2	14	3.1	16	3.1	15
Lys	6.0	22	5.4	23	4.6	20	6.3	28	6.0	31	6.6	32
His	2.2	7	1.6	7	2.3	10	1.8	8	1.5	8	1.7	8
Arg	5.6	17	6.1	26	7.4	32	4.8	21	5.6	29	4.8	23
Pro	11.8	53	4.5	19	4.9	21	4.3	19	4.6	26	3.9	19
Total††	438		429		431		442		521		483	
<i>M_r</i>	52 000		48 347		48 702		48 825		57 234		53 519	
<i>T_m</i> (°C)‡‡	95		60		60							

* The amino acid composition of the enzyme was estimated with a Hitachi 833 amino acid analyser after hydrolysis in 6 M HCl for 24 h at 110 °C.

† Calculated on the assumption that the enzyme had four Trp residues and a molecular mass of 52 kDa, and that asparagine (Asn) or glutamine (Gln) residues constituted 45% of the residues in the enzyme.

‡ From the gene structure [14].

§ From the gene structure [35,36].

|| From the gene structure [41].

¶ From the gene structure [42].

** From the gene structure [43].

†† Total residues.

‡‡ Temperature at which the enzyme was 50% inactivated in 30 min at pH 6.0 (*T. caldophilus* enzyme) and pH 6.5 (*Anabaena* and *E. coli* enzymes), as a measure of thermostability.

an assumed molecular mass of 52 kDa, the molar percentage for each amino acid and the most likely number of residues for each amino acid are shown in Table 3. AGPases from *T. caldophilus* GK24 (this study), the cyanobacterium *Anabaena*, *E. coli*, the potato small subunit, the potato large subunit and rice have almost identical amino acid compositions. However, *T. caldophilus* GK24 AGPase differs markedly from the mesophile and plant AGPases in its higher pI (7.4% as sum of Asp + Glu), Pro content (11.8%), Ala + Gly contents (19.9%) and its N-terminal amino acid sequence (Figure 3). These findings indicate the difference in primary structure of *T. caldophilus* GK24 AGPase from others. However, they take similar folded conformations, as their structural parameters derived from the amino acid composition are similar.

In summary, the thermostable AGPase was first purified to homogeneity from a thermophilic bacterium, *T. caldophilus* GK24. The activity of purified thermostable AGPase was maximal at 73–78 °C and stable at up to 95 °C for 30 min at pH 6.0. Other characteristics, including the molecular mass (52 kDa), the subunit structure (homotetrameric), the kinetic properties and the sequences of the N-terminus and internal peptides, were quite similar to those of AGPases from other bacterial sources. These results enable us to study further molecular cloning, structure–function relationships and glycogen metabolism in *T. caldophilus* GK24 and the thermostability of the enzyme.

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