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Characterization of a hyperthermostable glycogen phosphorylase from *Aquifex aeolicus* expressed in *Escherichia coli*

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Abstract

The glycogen phosphorylase gene (*glgP*) of *Aquifex aeolicus* (*Aae*), a hyperthermophilic bacterium, was cloned and expressed in *Escherichia coli* and the characteristics of the expressed enzyme were examined. The recombinant enzyme was purified to homogeneity by heat-treatment at 70 °C for 15 min to denature the contaminating *E. coli* proteins, followed by Ni–NTA agarose column chromatography to selectively trap the His-tagged enzyme. The purified enzyme gave a single band on SDS–PAGE with a molecular mass of approximately 80 kDa. The enzyme displayed optimal activity at pH 6.5 and was stable in the pH range from 4.0 to 10.0. The temperature at which optimal enzyme activity was observed was 100 °C and the enzyme retained 66% of its original activity after heating at 100 °C for 30 min. Kinetic studies using the purified enzyme demonstrated that the smallest primer molecule accepted for catalysis in the synthetic direction was maltotriose (G3) and that the smallest effective substrate for the reverse process, phosphorolysis, was maltotetraose (G4). The K_m and k_{cat} values were determined for various oligosaccharides (G3–G7) in both synthetic and phosphorolytic reactions and, remarkably, a maximum degree of specificity was observed toward substrates in the phosphorolytic direction.

Keywords: *Aquifex aeolicus*; Hyperthermophile; Glycogen phosphorylase; Thermostable enzyme

1. Introduction

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the mobilization of glucose-1-phosphate from glycogen that provides a readily useable source of energy. Glycogen phosphorylases have been isolated from a wide variety of organisms, including bacteria, yeast, plants, amphibians and mammals. The mammalian glycogen phosphorylases are regulated by phosphorylation, by phosphorylase kinase (EC 2.7.1.38) and by AMP. In contrast, the enzymes isolated from *Escherichia coli* and potato are neither phosphorylated nor controlled by AMP [1–3]. Unlike the bacterial and plant phosphorylases, the glycogen phosphorylase from yeast is regulated through allosteric mechanisms [4]. Glycogen phosphorylases have also been cloned and expressed from several mesophilic sources including *E. coli* [5], yeast [6], fungus [7] and rabbit muscle [8]. Although, significant progress has been made in regard to the detailed structural and functional characterization of glycogen phosphorylases from mesophilic origins, little is known about those from hyperthermophilic organisms.

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Hyperthermophiles are defined as organisms that grow optimally at temperatures of 80 °C or more. Thermophilic bacterium presents a profitable source for the isolation of highly thermostable enzymes whose biochemical and structural analyses may be extremely helpful in the task of unraveling the molecular basis of thermostability. In addition, the thermophilic enzymes produced by these types of organisms may prove to be valuable catalysts in various industrial applications. Although glycogen-like polysaccharides have been identified from several thermophilic bacteria [9], the only thermostable α-glucan phosphorylases reported to date have been isolated from the thermophilic bacteria *Thermus thermophilus* [10], *Thermus aquaticus* [11] and *Thermotoga maritima* [12].

Aquifex aeolicus (*Aae*) is a hyperthermophilic, hydrogen-oxidizing, microaerophilic, obligate chemolithoautotroph [13], representing the lowest branch in the phylogeny of bacteria [14,15]. This extremophile has recently generated interest among researchers as a useful source of thermostable enzymes [16–18]. The complete genome sequence of *A. aeolicus* has recently been determined [19] and found to contain 1512 open reading frames (ORFs), one of which encodes a putative glycogen phosphorylase (*glgP*) of 692 amino acids in length (GenBank accession; AE000704). To date there have been no reports of the isolation of this enzyme from the hyperthermophile *A. aeolicus*, a bacterium which has a growth-temperature maximum near 95 °C. Therefore, we cloned and expressed the *glgP* gene from *A. aeolicus* in *E. coli* and characterized the properties of the recombinant enzyme to examine the use of the enzyme for the synthesis of glucose-1-phosphate [20].

2. Materials and methods

2.1. Bacterial strains and plasmids

The genomic DNA of *A. aeolicus* was kindly provided by Professors K.O. Stetter and R. Hubber from the University of Rosenburg, Germany. Topo-XL TOP 10 [F$^-$ *mcr* AΔ (*mrr-hsd*RMS-*mcr*BC)φ80*lac*ZΔM15 Δ*lac*X74 *rec*AI *deo*R *ara*D139 Δ(*ara-leu*)7697*gal*U *gal*K *rps*L (*str*R) *end*AI *nup*G] was used as the host for the *glgP* gene from *A. aeolicus* (TOPO® XL cloning kit, Invitrogen, USA). *E. coli* BL21 [DE3-gold, RP and RIL] [*hsd* (*clts* 867 *indl sam* 7 *nin5lac UV5-T7 gene 1*] was used as the host for the recombinant plasmid harboring the pET28b (+) vector (Novagen, Madison, WI, USA) and for the subsequent expression step. Recombinant DNA techniques as described by Sambrook et al. [21] were employed for the DNA manipulations.

2.2. Construction of the glgP gene

The nucleotide sequence of the open reading frame denoted as the *glgP* gene was retrieved from GenBank with the accession number AE000704 (g2983305). The gene was amplified using the primers 5′CATATGCATATGGAAGAAGAAAAAGTAAAA-GAGG-3′ and 5′GGAATTCATCCCTCCTCCTTTA-AAATTTT-3′ which contained *Nde1* and *EcoR1* restriction sites, respectively (underlined). The expected 2079 bp fragment was obtained by using DNA polymerase (KOD-plus, Toyobo Co., Osaka, Japan) and a PCR program with 25 cycles comprising the following steps: 98 °C for 1 min; 55 °C for 1 min; and 68 °C for 2.3 min. The restriction enzyme sites were constructed at the ends of the primers in such a way that an N-terminal hexahistidine was able to be attached along with the *glgP* gene during expression. The amplified DNA fragment of the *glgP* gene was cloned into a pCR-TOPO-XL vector using a pCR-TOPO-cloning kit according to the protocol of the supplier (Invitrogen, USA). The resulting recombinant plasmids, AF-*glgP*-TOPO-XL, were extracted using a QIA Miniprep kit (GIAgen, Germany) and were sequenced to ensure that the nucleotide sequences were identical with those of the *glgP* gene. DNA sequencing was performed with an Applied Biosystems 310 Genetic Analyzer using a Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, USA). The sequence data were analyzed using the GENETYX computer program (Software Development Co., Tokyo, Japan).

2.3. Cloning and expression of the glgP gene in E. coli

The *glgP* gene was excised from the recombinant plasmid AF-*glgP*-TOPO-XL using the restriction enzymes *Nde1* and *EcoR1*, and it was then recovered

by ethanol precipitation before ligation to a pET28b (+) vector, which had been previously digested with the same pair of restriction enzymes. The ligation process was performed overnight at a temperature of 16 °C using a ligation kit, High T4 DNA ligase (Toyobo). *E. coli* BL21 [DE3-gold, RP and RIL] competent cells were transformed by electroporation with the ligated plasmids. The positive colonies from the transformed cells grown on LB medium containing kanamycin (50 μg/ml) were screened by colony PCR using vector specific primers (T7 promoter and T7 terminator primer) and these were used to prepare plasmid DNA on a small scale. One pET-AFGP clone was selected and it was confirmed by sequencing. For protein expression, the *E. coli* cells harboring plasmid pET-AFGP were grown in LB medium containing kanamycin (50 μg/ml) at 37 °C. After shaking at 37 °C until the absorbance at 600 nm (A600) reached a level of ∼0.6, IPTG was added to give a final concentration of 1.0 mM. After induction for 4 h, the *E. coli* cells were harvested by centrifugation, suspended in 50 mM Tris–HCl buffer (pH 7.0), and then disrupted by sonication (5 cycles consisting of 15 s pulses at 35% maximum output with 15 s rests between pulses). After centrifugation (12,000 rpm for 10 min), the supernatant was removed and this material was used as a crude extract.

2.4. Purification of the enzyme

Unless noted otherwise, the purification steps described below were performed at room temperature. To denature the *E. coli* proteins, the crude extract was heat-treated at 70 °C for 15 min and then the denatured host proteins were pelleted by centrifugation at 10,000 rpm for 20 min. The clear supernatant which contained the glycogen phosphorylase (20 ml) was mixed with Ni–NTA–agarose resin (2 ml) and kept on ice for 20 min. The resin bound to the target protein was then packed onto a 2 ml column connected to a FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) and the unbound proteins were washed off the column with 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM imidazole at a flow rate of 1 ml/min. The elution steps were performed using a linear concentration gradient from 10 to 250 mM imidazole in 50 mM Tris–HCl (pH 8.0) buffer. The fractions possessing glycogen phosphorylase activity were pooled together (to give a total volume of 14 ml) and dialyzed overnight against 2 l of 20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 6.5) at 4 °C.

2.5. Analysis of the purified protein

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [22] using 10% acrylamide gel slabs. Samples were boiled for 4 min in the presence of 0.01 M mercaptoethanol and 1% (w/v) SDS. Proteins were stained with 1% (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/ water (50:10:40, v/v) and they were destained in methanol/acetic acid/ water (30:10:60, v/v). A 10 kDa protein ladder (LIFE TECHNOLOGIES, GIBCO BRL, Rockville, USA) was used as a molecular weight marker.

2.6. Assay of glycogen phosphorylase activity

Glycogen phosphorylase activity was determined in the direction of oligosaccharide synthesis (assay A) and also in the degradation (phosphorolysis) direction (assay B).

Assay A: The production of inorganic phosphate from soluble starch and glucose-1-phosphate was measured using the method described by Saheki et al. [23] with slight modification. A reaction mixture (250 μl) containing 50 mM MOPS buffer (pH 6.5), 0.4% soluble starch, 10 mM glucose-1-phosphate and an appropriate amount of enzyme was incubated at 37 °C for 15 min. The reaction was stopped by the addition of 1 ml of 250 mM glycine–HCl buffer (pH 3) followed by rapid cooling in ice water. After cooling, 125 μl of 1% ammonium molybdate in 25 mM H_2SO_4 and 125 μl of 1% ascorbic acid in 0.05% $KHSO_4$ were added to the mixture. The resulting mixture was incubated at 37 °C for 30 min, then the absorbance was measured at 700 nm. One unit of enzyme activity is defined as the amount of the enzyme producing 1 μmol of phosphate in 1 min.

Assay B: Enzyme activity in the direction of oligosaccharide degradation (phosphorolysis) at 37 °C was measured in a coupled assay as described by Schinzel and Palm [24] with minor modification. The reaction mixture contained the following reagents in a final volume of 500 μl: 50 mM MOPS buffer (pH 6.5), 0.5% soluble starch, 1 mM $MgCl_2$, 5 mM NaH_2PO_4,

2.5 μM glucose 1-6-diphosphate, 0.015% NADP, 0.5 unit of glucose-6-phosphate dehydrogenase, 0.5 unit of phosphoglucomutase, and an appropriate amount of enzyme. The reaction was started by the addition of the enzyme and the increase in absorbance at a wavelength of 340 nm, due to the formation of NADPH, was followed continuously using a Beckman DU-650 UV-Vis spectrophotometer for 30 min at 37 °C. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of glucose-1-phosphate in 1 min at 37 °C.

2.7. Influence of pH and temperature

The activity of the purified enzyme at temperatures ranging from 0 to 100 °C was determined in the synthetic direction by using 5 mM maltopentaose and 10 mM glucose-1-phosphate as the substrates in 50 mM MOPS buffer (pH 6.5). For determining the thermal stability of the purified enzyme, the enzyme was pre-incubated for 30 min in 50 mM MOPS buffer (pH 6.5) at temperatures ranging from 0 to 100 °C. After chilling the sample on ice, the remaining activity was determined by the standard assay procedure (assay A). In all cases, the incubations were carried out in closed Eppendorf tubes in order to prevent boiling of the solutions.

The effect of pH on the activity displayed by the enzyme was determined in the synthetic direction at 37 °C in 50 mM acetate (pH 3.8–5.7), 2-(*N*-morpholino) ethane sulfonic acid (MES) (pH 5.2–6.2), 3-(*N*-morpholino) propanesulfonic acid (pH 6.2–8.2), *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (pH 6.5–8.5), Tris–HCl (pH 7.2–9.0) or 3-[cyclohexylamino]-1-propanasulfonic acid (CAPS) (pH 9.4–11.4) buffers containing 5 mM maltopentaose and 10 mM glucose-1-phosphate as the substrates. To determine the pH stability of the purified enzyme, the enzyme was pre-incubated in the buffers described above for 30 min at 70 °C, then the remaining activity was determined using the standard assay procedure (assay A).

2.8. Studies of the kinetic parameters

The reactions were followed by incubating the purified enzyme with appropriate concentrations of substrate (maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose or starch) in 50 mM MOPS buffer (pH 6.5) at 37 °C. In the synthetic direction, the release of phosphate was measured at 700 nm as described in the assay procedure A. In the phosphorolytic direction, the formation of NADPH was monitored at 340 nm using a Beckman DU-650 UV-Vis spectrophotometer equipped with a temperature controlled cell holder for 30 min at 37 °C, as described in the assay procedure B. Initial rates of reaction were determined at five different substrate concentrations. Values for K_m and k_{cat} were obtained by using the nonlinear regression analysis computer program GRAFIT [25].

3. Results

3.1. Cloning, expression and purification of glycogen phosphorylase

As described in the Section 2, we used PCR to place *Nde1* and *EcoR1* restriction enzyme sites at the 5′ and 3′ ends of the *glgP* open reading frame, respectively, so that the entire open reading frame could be inserted within the multiclone site of the pET28b (+) plasmid, thereby creating the plasmid pET-AFGP. The *Nde*1 site was positioned so that hexahistidine could be attached to N-terminal glycogen phosphorylase during translation. Four hours after IPTG induction, the strain BL21-RIL (pGP1) was found to express a protein with a molecular mass of approximately 80 kDa (Fig. 1, lane 1) which was not present prior to induction and did not appear in extracts from the parent strain BL21 or BL21-RIL (pET-AFGP) (data not shown). The 80 kDa protein remained in the supernatant when crude extracts from BL21-RIL (pET-AFGP) were subjected to heating at 70 °C for 15 min, causing approximately 50% of the *E. coli* proteins to precipitate. Resistance to heat-treatment at 70 °C is a characteristic of many proteins from *A. aeolicus* and the presence of heat resistance strongly suggested that the 80 kDa protein was the desired *glgP* product. After the heat-treatment step, Ni–NTA agarose (QIAGEN) was employed to trap the His-tagged enzyme and the glycogen phosphorylase was eluted at an imidazole concentration of approximately 100 mM. A summary

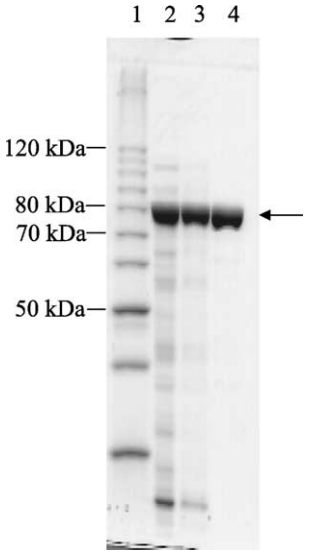

Fig. 1. SDS–PAGE of the glycogen phosphorylase at various stages of purification process. Molecular weight marker (lane 1); crude extract (lane 2); heat-treated crude extract (lane 3); and purified enzyme after the Ni–NTA column (lane 4).

of the purification process is shown in Table 1. Employing this process, approximately 11 mg of thermostable enzyme was purified from 1 l of culture. SDS–PAGE (Fig. 1, lane 4) indicated that the enzyme was homogenous and had a molecular mass of 80 kDa, consistent with the value of 81,981 Da that was calculated from the deduced amino acid sequence using the computer program ExPASY-Peptide Mass (http://kr.expasy.org/cgi-bin/peptide-mass).

3.2. Effect of pH

The activity of the enzyme was determined at various pH levels as described in the Section 2. Under the assay conditions used, the pH for the display of optimal activity for the recombinant glycogen phosphorylase at 37 °C was observed at pH 6.5 (Fig. 2A). The enzyme was stable between pH 4 and 10, and at a temperature of 70 °C it retained about 61% of its maximal activity at pH 10.4 (Fig. 2B).

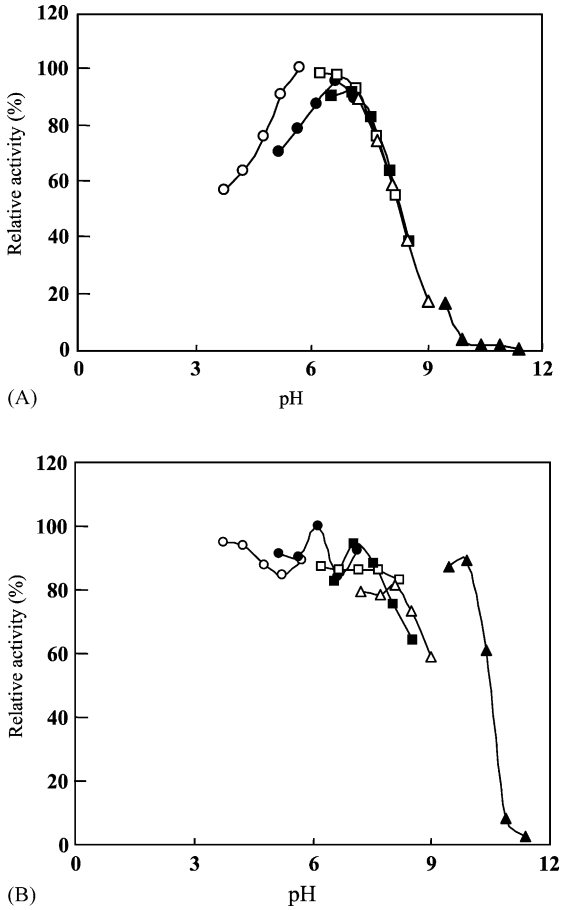

Fig. 2. Effect of pH on the activity (A) and stability (B) of the glycogen phosphorylase. The following buffers were used: 50 mM acetate (open circle), MES (solid circle), MOPS (open square), HEPES (solid square), Tris–HCl (open triangle), and CAPS (solid triangle).

Table 1
Summary of the purification of the glycogen phosphorylase from *A. aeolicus*

Purification step	Total protein (mg)	Total activity (unit)[a]	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extract	290	360	1.2	1.0	100
Heat precipitation	150	260	1.7	1.4	73
Ni–NTA–agarose	11	86	7.8	6.3	24

[a] Activity was determined at 70 °C, 15 min in the synthetic direction (assay A).

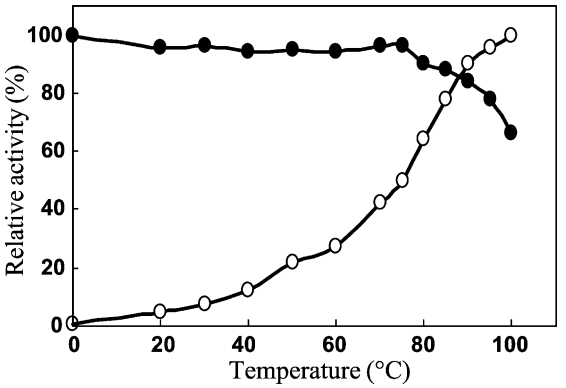

Fig. 3. Effect of temperature on the activity (open circle) and stability (solid circle) of glycogen phosphorylase. The temperature profile was determined at different temperatures using the standard assay (A) as described in Section 2 at the optimum pH 6.5 in 50 mM MOPS buffer. For the determination of thermal stability of the enzyme, the residual activity of the heat-treated enzyme was measured.

3.3. Effect of temperature

The atypical properties of this glycogen phosphorylase are its high optimum temperature and its high degree of thermostability. When the activity of the enzyme was tested at various temperatures, the recombinant glycogen phosphorylase showed less than 5% of its maximal activity at 20 °C and its activity increased with temperature up to 100 °C (Fig. 3). After incubating the purified enzyme for 30 min at selected temperatures between 0 and 100 °C, the relative activity of the enzyme was determined at 70 °C to examine the effects of heating. The enzyme retained about 84 and 66% of its original activity after heating at temperatures of 90 and 100 °C, respectively.

3.4. Substrate specificity and analysis of kinetic parameters

At 37 °C, various maltooligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) and soluble starch were used for measurements of the kinetic parameters at pH 6.5, in the direction of oligosaccharide synthesis as well as in the degradation direction. As shown in Table 2, in the presence of inorganic phosphate, the enzyme was able to phosphorolyze maltotetraose (G4) and larger oligosaccharides, while maltose and maltotriose were not degraded. In the presence of glucose-1-phosphate, maltotriose (G3) and larger oligosaccharides were effective acceptors for oligosaccharide synthesis, whereas glucose and maltose were not. These results indicate that the smallest substrate for the synthetic reaction is maltotriose (G3) and the smallest substrate for the phosphorolytic reaction is maltotetraose (G4). The K_m and k_{cat} values were determined using glucose oligomers of various lengths (G2–G7) as the substrates to examine the effect of the length of the substrate on the activity of the enzyme. As shown in Table 2, in the synthetic direction, no significant differences were detected for the k_{cat} values of substrates higher than G3. However, K_m values decreased three-fold from G3 (1.8 mM)

Table 2
Relative substrate specificities of the glycogen phosphorylase for the synthetic and phosphorolytic reactions

Substrate	Substrate direction[a]			Phosphorolytic direction[b]		
	K_m (mM)	k_{cat} (s^{-1})[c]	k_{cat}/K_m (s^{-1}/mM)	K_m (mM)	k_{cat} (s^{-1})[c]	k_{cat}/K_m (s^{-1}/mM)
Maltose	–	–	–	–	–	–
Maltotriose	1.8	6.8	3.9	–	–	–
Maltotetraose	0.55	6.0	11	0.3	0.76	2.5
Maltopentaose	0.28	5.4	19	0.17	2.2	13
Maltohexaose	0.3	4.1	13	0.18	2.7	15
Maltoheptaose	0.38	4.4	11	0.19	2.3	12
Starch	0.12%	5.2		0.03%	1.5	

The reaction was carried out at 37 °C in 50 mM MOPS buffer (pH 6.5).
[a] $Gn + G\ 1-P \rightarrow G(n+1) +$ phosphate.
[b] $Gn +$ phosphate $\rightarrow G(n-1) + G\ 1-P$.
[c] Based on a molecular mass of 81,981 Da.

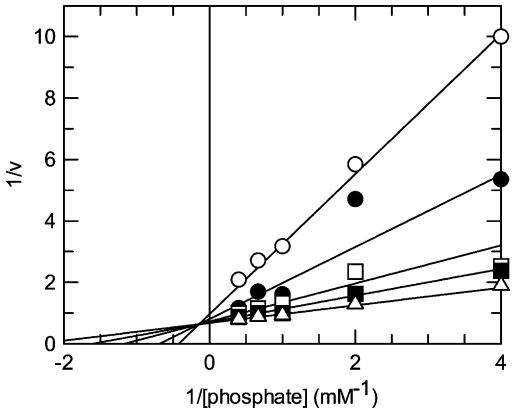

Fig. 4. The 1/V-1/[Pi] plot at various maltopentaose concentrations. Initial concentrations of maltopentaose are: 0.1 mM (open circle), 0.2 mM (solid circle), 0.4 mM (open square), 0.6 mM (solid square), and 1.0 mM (open triangle).

to G4 (0.55 mM) but no significant differences were observed in substrates higher than G4 in the synthetic direction. Moreover, in the phosphorolytic direction, the k_{cat} value for G5 ($13\,s^{-1}$) was five times higher than that observed for G4 ($2.5\,s^{-1}$) but the values were almost constant for substrates larger than G5. Furthermore, the K_m values were higher for the G4 substrate (0.30 mM) relative to G5 (0.17 mM) but the values were almost constant for substrates larger than G5 in the phosphorolytic direction. In terms of the relative specificities, K_m values for substrates in the synthetic direction were higher than the K_m values for substrates in the phosphorolytic direction, indicating that the specificity for the substrate is higher for the phosphorolytic reaction.

For the phosphorolytic reaction, the Lineweaver–Burk plot (1/V-1/[Pi]) is shown in Fig. 4, using various concentrations of maltopentaose. The lines intersect at a specific point in the second quadrant, indicating that the reaction followed a sequential mechanism. This result was in agreement with the mechanism of a phosphorylase previously described by Gold et al. [26] (viz. a random bi bi mechanism, a type of sequential mechanism).

4. Discussion

In this paper, we describe the characterization and identification of the *glgP* gene, expressed in *E. coli*, which encodes a thermophilic glycogen phosphorylase from the hyperthermophilic bacterium *A. aeolicus*. The amino acid sequence of the *A. aeolicus* glycogen phosphorylase was subjected to a BLAST search and it showed homology with maltodextrin phosphorylase from *Thermococcus litoralis* (45% identity [27]), α-glucan phosphorylase from *T. maritima* (43% identity [12]), α-glucan phosphorylase from *T. aquaticus* (39% identity [11]), α-glucan phosphorylase from *E. coli* (24% identity [28]), glycogen phosphorylase from *Dictyostelium discoidium* (24% identity [7]), glycogen phosphorylase from *E. coli* (23% identity [5]), and human liver glycogen phosphorylase (24% identity [29]). These enzymes belong to a large group of phosphorylases, comprising maltodextrin and glycogen phosphorylases from bacteria, eukaryotic unicellular organisms, as well as plants and mammals [3]. To our knowledge, this glycogen phosphorylase is the first enzyme of its kind to be purified and characterized from a hyperthermophilic bacterium, namely *A. aeolicus*. It has many properties in common with the other known phosphorylases. The *E. coli* enzyme, like most other known phosphorylases, is not able to degrade linear maltooligosaccharides with chain lengths shorter than maltopentaose and it cannot initiate the synthetic reaction with oligosaccharides shorter than maltotetraose [5]. In contrast, the glycogen phosphorylase from *A. aeolicus* showed a distinctly different substrate specificity, whereby maltotriose was observed to be the smallest substrate for glycogen synthesis and maltotetraose the smallest substrate for glycogen degradation (Table 2). Similar substrate specificities have also been reported by other researchers for the α-glucan phosphorylase from hyperthermophiles *T. thermophilus* [10] and *T. aquaticus* [11], and for the maltodextrin phosphorylase from *T. litoralis* [27].

The apparent molecular mass of thermostable glycogen phosphorylase by SDS–PAGE analysis was found to be 80 kDa. The *glgP* described here is the most thermophilic glycogen phosphorylase identified to date, with an optimum temperature of 100 °C for maximum enzyme activity and with 66% of activity remaining after boiling in water for 30 min. The α-glucan phosphorylases from other thermophilic sources, *T. thermophilus*, *T. maritima* and *T. aquaticus*, have optimum temperatures of 70, 75–80 and 80–85 °C, respectively [10–12]. Based on structural analyses, the major factors that stabilize proteins from

hyperthermophiles are the number of ionic bonds and the presence of salt bridges and hydrogen bonds between charged amino acids [30–32]. Consistent with this is the observation that arginine, lysine, aspartic and glutamic acids are the predominant amino acids present in the sequence of *A. aeolicus* glycogen phosphorylase. An efficient and relatively simple purification procedure yielded an enzyme which was more than 95% pure after passing the heat-treated enzyme through an affinity Ni–NTA agarose column. We have taken advantage of the enzyme's tolerance toward high temperatures during the purification process and have achieved a high level of enzyme purity through a simple heat-treatment of the cellular extract. The general concept of applying a heat-treatment step may prove useful in the purification of other recombinant proteins from this organism.

In conclusion, similar to other enzymes from the *A. aeolicus*, the recombinant glycogen phosphorylase reported herein is extremely thermostable enzyme. In addition to observed hyperthermostability, this enzyme showed the maximum specificity for the substrates in the phosphorolytic direction. These properties may prove this glycogen phosphorylase a good candidate as an industrially useable enzyme for the production of glucose-1-phosphate on a large scale.

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