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Carsten Raasch · Wolfgang Streit · Jürgen Schanzer
Miriam Bibel · Uwe Gosslar · Wolfgang Liebl

Thermotoga maritima AglA, an extremely thermostable NAD^+-, Mn^{2+}-, and thiol-dependent α-glucosidase

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Abstract The gene for the α-glucosidase AglA of the hyperthermophilic bacterium *Thermotoga maritima* MSB8, which was identified by phenotypic screening of a *T. maritima* gene library, is located within a cluster of genes involved in the hydrolysis of starch and maltodextrins and the uptake of maltooligosaccharides. According to its primary structure as deduced from the nucleotide sequence of the gene, AglA belongs to family 4 of glycosyl hydrolases. The enzyme was recombinantly expressed in *Escherichia coli*, purified, and characterized. The *T. maritima* α-glucosidase has the unusual property of requiring NAD^+ and Mn^{2+} for activity. Co^{2+} and Ni^{2+} also activated AglA, albeit less efficiently than Mn^{2+}. *T. maritima* AglA represents the first example of a maltodextrin-degrading α-glucosidase with NAD^+ and Mn^{2+} requirement. In addition, AglA activity depended on reducing conditions. This third requirement was met by the addition of dithiothreitol (DTT) or β-mercaptoethanol to the assay. Using gel permeation chromatography, *T. maritima* AglA behaved as a dimer (two identical 55-kDa subunits), irrespective of metal depletion or metal addition, and irrespective of the presence or absence of NAD^+ or DTT. The enzyme hydrolyzes maltose and other small maltooligosaccharides but is inactive against the polymeric substrate starch. AglA is not specific with respect to the configuration at the C-4 position of its substrates because glycosidic derivatives of D-galactose are also hydrolyzed. In the presence of all cofactors, maximum activity was recorded at pH 7.5 and 90°C (4-min assay). AglA is the most thermoactive and the most thermostable member of glycosyl hydrolase family 4. When incubated at 50°C and 70°C, the recombinant enzyme suffered partial inactivation during the first hours of incubation, but thereafter the residual activity did not drop below about 50% and 20% of the initial value, respectively, within a period of 48 h.

Key words *Thermotoga* · α-Glucosidase · Cofactor · NAD · Thermostability

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C. Raasch · W. Streit · J. Schanzer · M. Bibel · U. Gosslar · W. Liebl (✉)
Institut für Mikrobiologie und Genetik, Georg-August-Universität, Grisebachstr. 8, D-37077 Göttingen, Germany
Tel. +49-551-393795; Fax +49-551-393793
e-mail: wliebl@gwdg.de

Introduction

The hyperthermophilic bacterium *Thermotoga maritima* is characterized by a growth range between 55° and 90°C with an optimum at 80°C (Huber et al. 1986), making it one of the most thermophilic bacteria known to date. This organism degrades and fermentatively utilizes various organic compounds, including starch and its α-glucan polysaccharide constituents, amylose and amylopectin. In the course of analyzing the carbohydrate utilization enzymes of *T. maritima* MSB8, we have identified several different enzyme activities putatively involved in starch utilization and have isolated the corresponding chromosomal genes (Liebl et al. 1992, 1997; Huber and Liebl 1994; Heinrich et al. 1994; Bibel et al. 1998; Meissner and Liebl 1998).

α-Glucosidases are enzymes that hydrolytically cleave α-glucopyranosidic bonds in glucose-containing oligosaccharides. Typically, these enzymes do not degrade polymeric α-glucans like amylose or amylopectin, although there are a few exceptions (Rolfsmeier et al. 1998; Suzuki et al. 1992). They can be classified according to their substrate specificity. Most of the known α-glucosidase enzymes are exo-acting glycosidases that cleave the α-1,4-glycosidic bonds in maltose [*O*-α-D-glucopyranosyl-(1 → 4)-D-glucose] or other linear maltooligosaccharides (typical α-glucosidase activity), but some also are known which hydrolyze α-1,6-bonds of isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucose] or other branched maltooligosaccharides (oligo-1,6-glucosidase; isomaltase activity), or the α-glucosidic bonds of other glucose disaccharides, i.e., trehalose [*O*-α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside; trehalase activity] or nigerose [*O*-

α-D-glucopyranosyl-(1 → 3)-D-glucose]. Also, some α-glucosidases can hydrolyze the glycosidic bonds of sucrose [*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside; invertase activity], turanose [*O*-α-D-glucopyranosyl-(1 → 3)-D-fructofuranose], or melizitose [*O*-α-D-glucopyranosyl-(1 → 3)-*O*-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside]. An endo-acting group of α-glucosidases are able to attack circular maltooligosaccharides (cyclodextrins; cyclodextrinase activity). Finally, certain α-glucosidases are specific for phosphorylated disac-charide substrates (trehalose-6-phosphate or maltose-6-phosphate) formed during disaccharide internalization via phosphoenolpyruvate-dependent phosphotransferase uptake systems (6-phospho-α-glucosidase activity). Some of the known α-glucosidases combine more than one of these specificities; e.g., MalL of *Bacillus subtilis* has sucrase, isomaltase, and maltase activity (Schönert et al. 1998).

α-Glucosidases have been identified in many prokaryotic and eukaryotic organisms, but information about the occurrence and properties of these enzymes from hyperthermophiles is scarce, with the exception of reports about the properties of α-glucosidases of the hyperthermophilic Archaea *Thermococcus* sp. (Piller et al. 1996), *Pyrococcus furiosus* (Badr et al. 1994; Constantino et al. 1990), and *Sulfolobus solfataricus* (Rolfsmeier et al. 1998; Rolfsmeier and Blum 1995). None of these enzymes appear to have an essential requirement for the addition of a cofactor for activity. We have recently cloned and sequenced the *aglA* gene of *T. maritima* MSB8 (Bibel et al. 1998). Now we report the characterization of the enzyme encoded by this gene that represents the first example of an α-glucosidase from a hyperthermophilic bacterium. We decided to obtain the enzyme from a recombinant source because previous work had indicated a very low expression level of amylolytic enzymes in *T. maritima* (Liebl et al. 1992, 1997; Schumann et al. 1991; our unpublished data). The molecular cloning of the corresponding gene and its expression in *E. coli* was expected to avoid the problem of insufficient expression in the authentic host.

Initial attempts to purify the α-glucosidase of *T. maritima* MSB8 for further characterization turned out to be difficult because of rapid loss of enzyme activity during chromatographic purification steps and rapid inactivation during storage, but on the other hand provided indications that the α-glucosidase had an unusual cofactor requirement that had not been previously reported for other α-glucosidases. This phenomenon aroused our interest and prompted further purification and characterization efforts. In this communication we show that this enzyme requires NAD^+, Mn^{2+}, and a relatively high concentration of thiol-containing reducing agents such as dithiothreitol for full activity.

Materials and methods

Strains and plasmids

Recombinant DNA techniques and expression of the recombinant gene were performed in *Escherichia coli* JM83 (Yanish-Perron et al. 1985). *E. coli* strains were routinely grown in Luria–Bertani (LB) broth (Ausubel et al. 1987) supplemented with ampicillin ($100\,\mu g\,ml^{-1}$) or oxytetracyclin ($12\,\mu g\,ml^{-1}$) where appropriate. Recombinant clones with thermostable α-glucosidase activity were isolated from a gene library of *Thermotoga maritima* MSB8 (DSM 3109) (Ruile et al. 1997) as described previously (Bibel et al. 1998) by ligation of 3- to 6.5-kb partial *Sau*3A fragments of chromosomal DNA with *Bcl*I-digested vector pUN121 (Nilsson et al. 1983). Four recombinant *E. coli* JM83 clones with thermostable α-glucosidase activity were obtained. The plasmids of these clones, designated pTGL29, pTGL31, pTGL36, and pTGL46, had insert sizes of 4.2, 5.2, 4.5, and 2.5, respectively, with a common fragment of about 2.5 kb containing the α-glucosidase gene, *aglA*.

Clones expressing thermostable α-glucosidase activity were identified with the following plate assay. First, recombinant clones were grown on LB agar plates containing $12\,\mu g\,ml^{-1}$ oxytetracyclin and then transferred to fresh plates with a replica method. After growth at 37°C for 18 h, the replica plates were heated to 65°C for 15 min and the colonies were covered with filter paper disks soaked with 50 mM sodium phosphate buffer pH 7.0 containing 3 mM 4-methylumbelliferyl-α-D-glucoside (Sigma, Deisenhofen, Germany). Incubation was carried on for 5–15 min before looking for fluorescent colonies under UV (366-nm) illumination, and positive clones were recovered from the master plates. pBSKBE2.4 and pWBE2.4 contain the *aglA* gene on a 2.4-kb *Eco*RV(partial)–*Bam*HI fragment of pTGL29 cloned into *Eco*RV–*Bam*HI-cut pBluescript-SK (Stratagene) or *Sma*I-cut expression vector pWLQ2 (Liebl et al. 1992), respectively.

General DNA modification methods and sequence analysis

DNA manipulations and electrotransformation of *E. coli* were done according to standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). Computer analysis of nucleotide sequences and the deduced amino acid sequences was done with the programs DNASIS and PROSIS (v7.0; Hitachi Software, Tokyo, Japan). Similarity searches were performed with FASTA (v3.07; Pearson and Lipman 1988) and BLAST (Altschul et al. 1990). Pairwise alignments for the calculation of protein sequence similarities were created with the program GAP included in the University of Wisconsin Genetics Computer Group (UWGCG) software package for UNIX (v8.1; Devereux et al. 1984). Nucleotide sequences were from our own studies, from the EMBL or Genbank sequence libraries, and from The Institute of Genomic Research. Amino acid sequences were retrieved from the SWISS-PROT and PIR protein sequence databases.

Enzyme assays and analytical methods

Unless mentioned otherwise, the following standard α-glucosidase assay based on the enzymatic liberation of

nitrophenol from para-nitrophenyl-α-D-glucoside (pNP-α-Glc) was used. Assay mixtures (total volume, 300 µl) contained 30 µl 0.5 M Tris-HCl pH 7.0, 30 µl 10 mM $MnCl_2$, 30 µl 9 mM NAD^+, 15 µl 1 M dithiothreitol (DTT), and enzyme. After preequilibration of this mixture for 5 min at 60°C, the reaction was started by addition of 10 µl 0.1 M pNP-α-Glc. After 4–20 min at 60°C, the reaction was cooled on ice, mixed with 20 µl 0.5 M EDTA pH 8 and 680 µl water, and the absorbance was measured at 420 nm. The molar extinction coefficient of pNP under these conditions was $12000\,M^{-1}\,cm^{-1}$. One unit was defined as the amount of enzyme that released 1 µmol of pNP in 1 min under the specified conditions. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (1970).

The determination of enzyme activity versus pH was done with a 10-min assay at 60°C, using an enzyme concentration of about $4.2\,\mu g\,ml^{-1}$, in the presence of 0.9 mM NAD^+, 1 mM $MnCl_2$, and 50 mM DTT. The buffer systems used were 0.1 M sodium succinate (pH 5.5–7.0), 0.1 M Tris-HCl (pH 7.0–8.5), and 0.1 M 3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO; pH 9.0–9.5). The "temperature optimum" was determined with a 4-min assay at pH 7 in the presence of 0.9 mM NAD^+, 1 mM $MnCl_2$, and 50 mM DTT. In this case, the reaction was started by the addition of $5\,\mu g\,ml^{-1}$ of purified enzyme. Thermoinactivation data were obtained by incubating samples of recombinant AglA ($0.22\,mg\,ml^{-1}$) dissolved in 50 mM Tris-HCl (adjusted to pH 7 at 60°C) at various temperatures; 10-µl aliquots were drawn after various periods of time, and the residual activity was measured under the standard assay conditions.

For the qualitative analysis of α-glucosidase activity with various substrates, assay mixtures containing 50 mM Tris-HCl pH 7, 0.9 mM NAD^+, 1 mM $MnCl_2$, 50 mM DTT, 0.25% (w/v) of substrate, and enzyme at about $63\,\mu g\,ml^{-1}$ were incubated at 60°C for 5 h. The formation of reaction products was analyzed with thin-layer chromatography (TLC). TLC was done on 0.2-mm silica gel-coated aluminum sheets (type 60; Merck, Darmstadt, Germany), developed twice with 1-propanol/ethyl acetate/H_2O (6:1:3, v/v/v) or acetonitrile/H_2O (85:15, v/v) at room temperature. Carbohydrate spots were visualized by spraying the chromatograms with diphenylamine-aniline reagent [1% (w/v) diphenylamin and 1% (v/v) anilin in acetone, mixed with 0.1 vol. 85% phosphoric acid just before use] and incubating the plates at 140°C for 12 min.

Purification of recombinant α-glucosidase

For the preparation of AglA, a recombinant *E. coli* JM83 strain with plasmid pWBE2.4 was grown with aeration at 37°C in LB broth containing $100\,\mu g\,ml^{-1}$ ampicillin. In the exponential growth phase, at OD_{600nm} = 0.7, 0.2 mM isopropyl thiogalactoside (IPTG) was added, and incubation was continued for 18 h. Cells were harvested, washed with 20 mM Tris-HCl pH 8.0, resuspended in a small volume of the same buffer, and disrupted by repeated passage through a French pressure cell (American Instrument, Silver Spring, MD, USA) at 6.9 MPa. After centrifugation (40000 × *g*, 30 min, 4°C), the cleared homogenate was incubated at 80°C for 15 min. Denatured host cell proteins were sedimented (40000 × *g*, 30 min, 4°C), and the supernatant was subjected to anion-exchange chromatography on a Source 15 Q (Pharmacia, Freiburg, Germany) column, employing a linear 0.1 to 0.4 M NaCl gradient in 20 mM Tris-HCl pH 8.0 for elution. Fractions containing thermostable α-glucosidase activity were pooled, dialyzed against 50 mM Tris-HCl pH 7.0, and concentrated by ultrafiltration.

It was difficult to quantify the effectivity of the purification steps because AglA proved to be relatively unstable. The specific activity measured directly after the heat precipitation step (estimated purity according to SDS-PAGE analysis, >85%) was $11.5\,U\,mg^{-1}$, but dropped to $8.8\,U\,mg^{-1}$ after anion-exchange chromatography even though SDS-PAGE demonstrated that obviously several contaminating protein bands had been separated from the enzyme band (estimated purity, >95%). We have repeatedly observed significant activity loss during storage (especially during the first few days at 4°C or −20°C) and after freeze-thaw cycles. Various attempts to circumvent this problem (storage temperature, stabilizing additives, etc.) were not successful (not shown).

Results

Sequence analysis of the α-glucosidase and its gene aglA

The nucleotide sequence of the *aglA* gene, which was isolated in a previous study from a *T. maritima* MSB8 gene library (Bibel et al. 1998; see Materials and methods), was analyzed in detail. The sequence was found to contain a sequencing error that has the consequence that the open reading frame encoding AglA is 21 codons longer than originally published by Bibel et al. (1998). Comparison of *aglA* (EMBL database accession number AJ001089) and surrounding sequences with sequence data of the *T. maritima* genome sequencing project available from the database of The Institute of Genome Research (TIGR, Rockville, MD, USA; Nelson et al. 1999) and with other sequences available in our group revealed that the gene is located within a starch/maltodextrin utilization gene cluster (Fig. 1) containing the following genes (relative gene orientation indicated by arrows in brackets): *amyA*(←)-*malE*(→)-*malF*(→)-*malG*(→)-*aglB*(→)-*aglA*(→). The maltodextrin-binding protein gene *malE* and the α-amylase gene *amyA*, which is located upstream of but divergently oriented to *malE*, were described by us previously (Liebl et al. 1997).

The AglA-encoding reading frame translates into a polypeptide of 480 amino acids with a calculated molecular mass of 55046 Da. The codon usage for *aglA* mirrors the

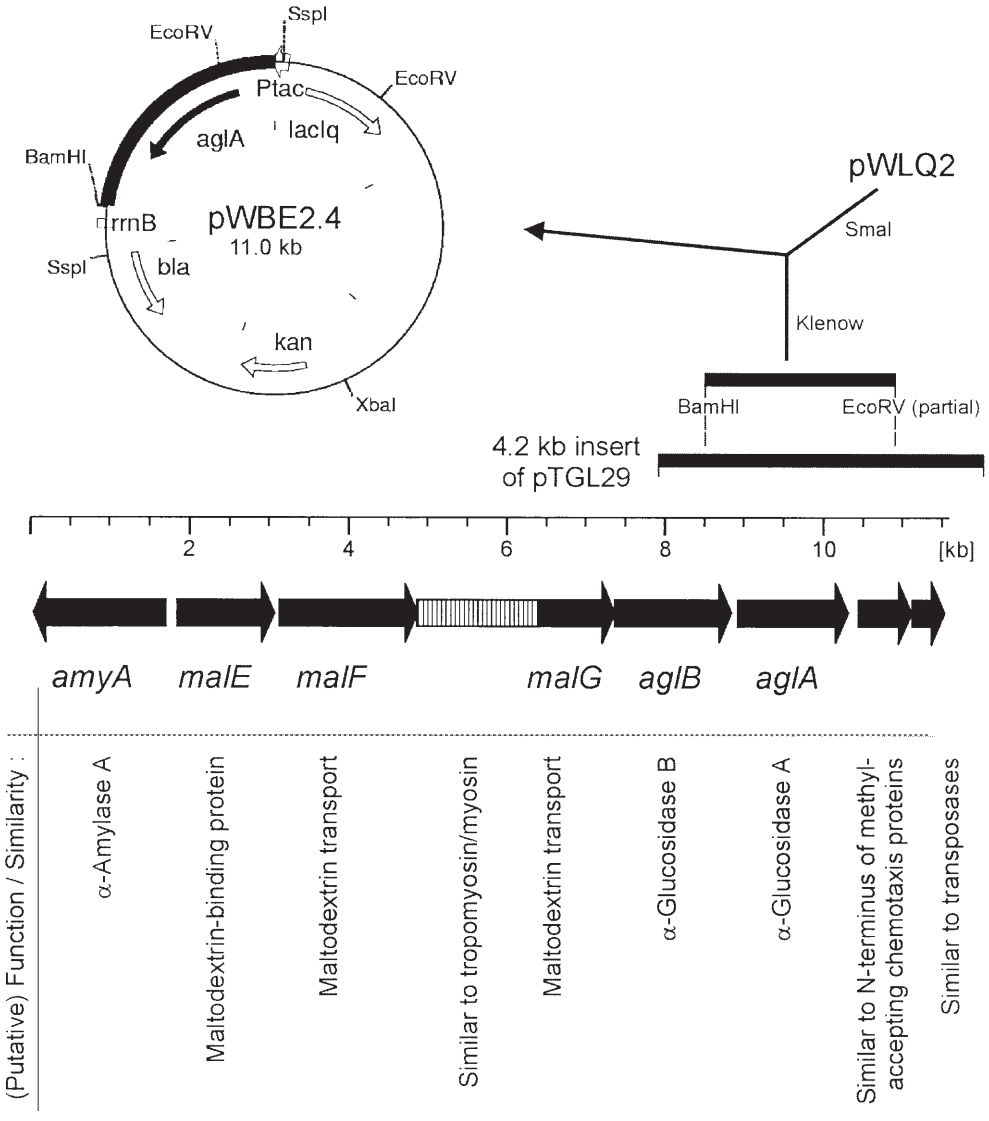


Fig. 1. Schematic representation of a region of the *Thermotoga maritima* MSB8 chromosome containing a cluster of genes encoding enzymes of starch/maltodextrin utilization. The gene order was assembled from sequences available in our group (Liebl et al. 1997; Bibel et al. 1998; this work; unpublished data) and sequence data of the *T. maritima* genome sequencing project available from the database of The Institute of Genome Research (TIGR, Rockville, MD, USA)

typical codon usage in *T. maritima* genes (data not shown). Five nucleotides upstream of the start codon we found a potential ribosome-binding sequence (5′-...GGAGGTG...-3′) with perfect complementarity to the 3′-end of the 16*S* rRNAs of both *T. maritima* and *E. coli* (3′-UCUUUCCUCCACU...5′ and 3′-AUUCCUCCACU...5′, respectively). A G+C-rich inverted repeat (5′-...AAAGGGGCCTGAAAGGCCCCTTT...-3′) about 12 bp downstream of the coding sequence could represent a transcription termination signal.

According to the amino acid sequence similarity-based classification of glycosyl hydrolases (Henrissat 1991; Henrissat and Bairoch 1993), AglA belongs to glycosyl hydrolase family 4. This enzyme family contains oligosaccharidases with various specificities, i.e., α-galactosidases and phospho-β-glucosidases. More recently also phospho-α-glucosidases and now the α-glucosidase of *T. maritima* were assigned to this family (Bibel et al. 1998; Thompson et al. 1998; this work). Thompson et al. (1998) have recently demonstrated that three acidic residues are essential for catalytic activity of the family 4 enzyme GlvA of *Bacillus subtilis*. These residues (Asp41, Glu111, and Glu359 according to the numbering of GlvA), which are conserved in family 4, are also present in *T. maritima* AglA (Asp39, and Glu113, Glu391, respectively) and are probably of similar importance for the function of this enzyme.

Purification and analysis of the cofactor requirement of recombinant AglA

To facilitate enzyme purification, the *aglA* gene was placed under the control of the efficient *E. coli* p_{tac} promoter. For this purpose, a 2.4-kb *aglA*-bearing fragment of *T. maritima* DNA was obtained by *Bam*HI and *Eco*RV (partial) digestion of plasmid pTGL29. After Klenow treatment to fill in the *Bam*HI-generated 3′-recessed strand, the fragment was inserted into the *Sma*I restriction site of expression vector pWLQ2. Transformation of the resulting

plasmid, designated pWBE2.4 (see Fig. 1), into *E. coli* JM83 yielded the recombinant strain used for preparation of crude enzyme and large-scale α-glucosidase purification. For some experiments *E. coli* JM83 bearing pBSKBE2.4, which is a high copy number plasmid bearing the same 2.4-kb *Bam*HI–*Eco*RV fragment as pWBE2.4, was used as indicated next.

During initial attempts to purify recombinant AglA with protein chromatographic methods, we could not detect α-glucosidase activity in any of the eluate fractions. This result indicated that one (or more) cofactors may have been separated from the enzyme during chromatography. After a series of experiments performed as described next, we finally found that AglA needs three different additives, i.e., Mn^{2+}, NAD^+, and DTT (or another reducing agent) for full activity.

Using crude enzyme [prepared by heat treatment of a lysate of *E. coli* JM83(pBSKBE2.4) at 75°C for 15 min] in an assay system containing 50 mM Tris-HCl pH 7 and 3.3 mM pNP-α-D-glucoside, we first analyzed the possible effect of metal ions on enzyme activity. Enzyme activity was completely abolished by the addition of 2 mM EDTA, suggesting a role for a divalent metal cation for activation. Of the various metals tested (all as chloride salts at 1 mM concentration), Mn^{2+} and to a lesser extent Co^{2+} and Ni^{2+} had an activating effect. In the presence of 1 mM Mn^{2+}, Hg^{2+} at 0.1 mM almost completely abolished pNP-α-glucoside cleavage (2% relative activity).

The high sensitivity of the enzyme against Hg^{2+} (see earlier) indicated that AglA may contain essential sulfhydryl groups and thus prompted us to determine if reducing agents would exert an effect on α-glucosidase activity. Using crude enzyme (preparation and assay conditions as mentioned earlier) in the presence of 1 mM $MnCl_2$, we found that dithiothreitol (DTT) significantly enhanced enzyme activity. Interestingly, relatively high concentrations of 50 mM DTT (0.77%) or more were needed for maximum activity.

Despite the addition of the now known activators $MnCl_2$ and DTT to the assay mixtures, chromatographic protein purification attempts, using Q-Sepharose HP and Phenyl-Sepharose HP columns, still yielded no active eluate fractions. Because a protein with the same size as the main expressed protein of the recombinant clone was detected in the eluate fractions (data not shown), however, it was suspected that the enzyme had a further cofactor requirement. The activity of the enzyme present in the chromatographic fractions could be restored by the addition of a small amount of *E. coli* JM83 crude extract. The reactivating compound was also present in the filtrate obtained by centrifugation-aided ultrafiltration (cutoff, 3 kDa; Amicon) of the *E. coli* JM83 crude extract. Further experiments demonstrated that the compound was not chloroform extractable and that it was insensitive against degradation with proteinase K (data not shown). These results demonstrated that the compound was not *Thermotoga* specific, that it was not encoded by the recombinant DNA fragment that carried the *aglA* gene, and that it was of low molecular mass. Of a number of substances (folic acid, riboflavin, flavin mononucleotide, flavin adenine dinucleotide, ATP, thiamine pyrophosphate, biotin, NAD^+, $NADP^+$, NADH, NADPH) subsequently tested in an assay system containing 1 mM $MnCl_2$, 50 mM DTT, and cofactor-depleted AglA (inactive enzyme eluted with a salt gradient from a Q-Sepharose HP column), only the addition of NAD^+ resulted in enzyme activation.

To further characterize the unusual cofactor dependency of AglA, additional experiments were performed using purified recombinant enzyme prepared from *E. coli* JM83(pWBE2.4) as described in Material and methods (Fig. 2). The enzyme preparation obtained contained one contaminating band that was not removed in various attempts. However, the enzyme was sufficiently pure (>95%) for further characterization. Each of the three activators NAD^+, Mn^{2+}, and DTT appears to be equally important for α-glucosidase activity because enzyme activity was generally less than 2.5% of the maximum if only one of them was missing (Fig. 3). The effect of increasing concentrations of each of the activators on enzyme activity was determined at 60°C in 50 mM Tris-HCl pH 7 in the presence of optimal concentrations of the other activators (Figs 4, 5). The saturating cofactor concentrations needed to achieve highest activity were about 0.9 mM NAD^+, 1 mM $MnCl_2$, and 50 mM DTT. These numbers were used to establish an optimized assay protocol for further enzyme characterization.

The stimulating effect of DTT indicated that a reducing agent was needed for α-glucosidase activity. A possible substitute for DTT was 2-mercaptoethanol. This compound was most effective at about 600 mM or more (see Fig. 5).

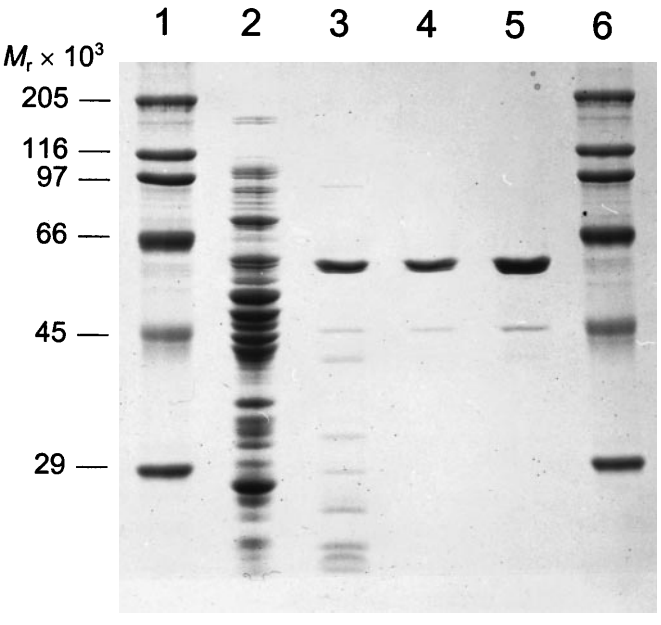


Fig. 2. Purification steps of recombinant α-glucosidase analyzed by SDS-PAGE (10%). *Lanes 1* and *6*, molecular weight markers; *lane 2*, crude extract of *E. coli* JM83(pWBE2.4) (15 μg); *lane 3*, crude extract after heat precipitation (1 μg); *lane 4*, pooled fractions after Source 15Q anion-exchange chromatography (1 μg); *lane 5*, pooled fractions after Superdex 200 gelfiltration chromatography (3 μg)

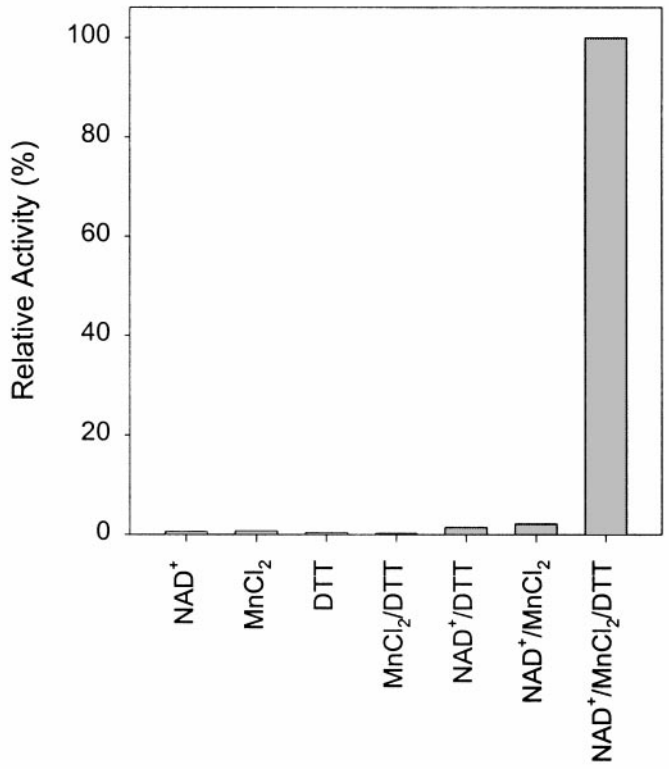


Fig. 3. Effect of addition of NAD^+, $MnCl_2$, and DTT on the activity of purified AglA. Concentrations of cofactors were NAD^+, 0.9 mM; $MnCl_2$, 1 mM; DTT, 50 mM

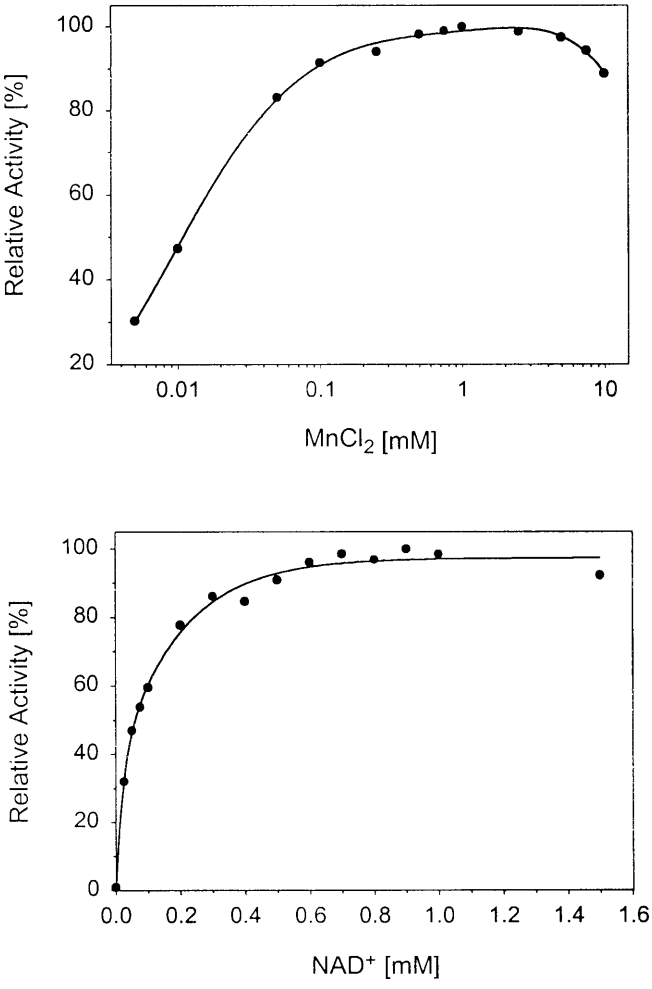


Fig. 4. Effect of the concentration of Mn^{2+} (*upper graph*) and NAD^+ (*lower graph*) on the activity of purified AglA. The effect of $MnCl_2$ was measured in the presence of 0.9 mM NAD^+ and 50 mM DTT. In the case of changing NAD^+ concentrations, the levels of $MnCl_2$ and DTT were kept constant at 1 mM and 50 mM, respectively

Replacement of DTT by L-cysteine also led to enzyme activation, with a maximum effect at a L-cysteine concentration of 60 mM or more. However, maximum activation observed with 60 mM L-cysteine was about threefold lower than with 50 mM DTT (Fig. 5). It was surprising that extremely high concentrations of either DTT (about 50 mM), L-cysteine (about 60 mM), or 2-mercaptoethanol (about 600 mM) were necessary for activation. Thus the question arose whether other properties of these additives aside from merely reducing power played a role in activation. We attempted to use structurally similar but nonthiolic compounds as substituents; i.e., DTT was partially replaced by erythritol or glycerol, and 1,2-ethanediol and ethanol were tried as substitutes for 2-mercaptoethanol. For this purpose, assay mixtures contained 50 mM Tris-HCl pH 7, 1 mM $MnCl_2$, 0.9 mM NAD^+, a suboptimal level (5 mM) of the reductant DTT, enzyme at 2.2 $\mu g\,ml^{-1}$, and 5 to 500 mM (5 mM–50 mM for erythritol) of the hydroxyl-containing substances listed. It was not possible to reduce the DTT concentration by partial substitution with one of these substances without a simultaneous drop in enzyme activity. It is not yet clear why such a high concentration of reducing agent is needed for maximum activation.

The effects of various divalent metal ions on AglA activity are summarized in Table 1. Using purified recombinant enzyme in the presence of 0.9 mM NAD^+ and 50 mM DTT, the stimulatory effect of Mn^{2+} was confirmed, but it was not possible to determine the effects of Fe^{2+}, Co^{2+}, or Ni^{2+} because a turbid precipitate was formed in the presence of DTT (even at a low concentration of 5 mM). However, by using a heat-treated (85°C, 15 min) crude extract of *E. coli* JM83(pWBE2.4) as the source of enzyme (this contains activating compounds, albeit not at optimal concentrations) we circumvented the need to add DTT and showed that Co^{2+} and Ni^{2+} were also stimulatory (Table 1), but the data with Fe^{2+} were still not interpretable as a result of interference with the assay.

Other enzyme properties and substrate specificity of AglA

The size of the purified enzyme as determined by SDS-PAGE analysis was about 58 kDa, which is slightly larger than the size calculated from the primary structure (55 kDa). During analytical gel permeation chromatography on a Superdex 200 HiLoad 16/60 column (Pharmacia) equilibrated with 50 mM Tris-HCl pH 7

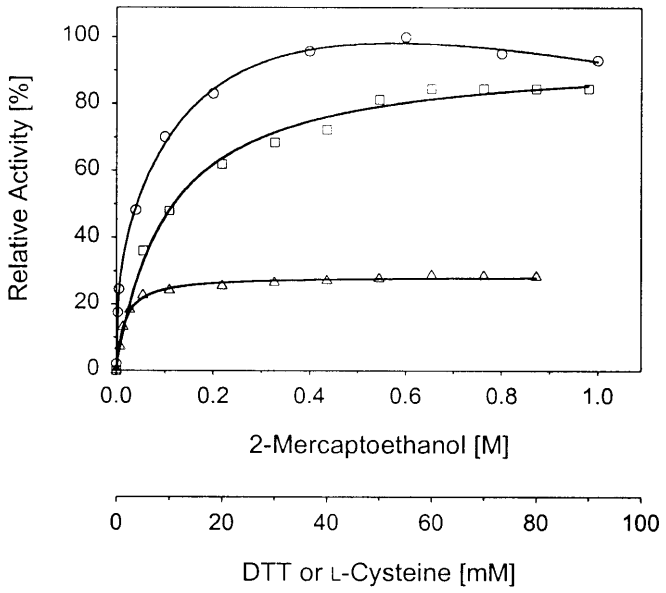


Fig. 5. Effect of DTT, L-cysteine, and 2-mercaptoethanol on AglA activity. Increasing amounts of DTT (*squares*), 2-mercaptoethanol (*circles*), or L-cysteine (*triangles*) were added to standard assay mixtures containing the cofactors NAD^+ and $MnCl_2$ at optimum concentrations of 0.9 mM and 1 mM, respectively

Table 1. Effect of divalent metal ions on the activity of recombinant AglA

Additive (1 mM)	Relative activity (%): Purified AglA	JM83(pWBE2.4) crude extract with AglA activity[a]
	Assay conditions: 50 mM Tris-HCl pH7 0.9 mM NAD^+ 50 mM DTT	Assay conditions: 50 mM Tris-HCl pH7 0.9 mM NAD^+ no DTT
None	0	13
Mg^{2+}	1.1	13
Ca^{2+}	3.1	15
Sr^{2+}	0.2	13
Ba^{2+}	0.2	13
Mn^{2+}	100[b]	100[b]
Fe^{2+}	n.d.[c]	n.d.
Co^{2+}	n.d.	59
Ni^{2+}	n.d.	25
Cu^{2+}	0.1	5.4
Zn^{2+}	3.1	14

[a] Note the high basal level of AglA activity caused by the presence of a suboptimal amount of stimulating compounds (metal ions, reducing substances, NAD^+) in the crude extract

[b] The specific activities for purified AglA and crude extract with AglA activity under the specified assay conditions were 8.5 U/mg and 2.8 U/mg, respectively

[c] n. d., no reliable data due to incompatibility of metal ions with the assay conditions

containing 150 mM NaCl, the enzyme eluted with a volume corresponding to a size of about 110 kDa. Similar results were obtained (102–120 kDa) when gel filtration experiments were done in the presence of 1 mM $MnCl_2$, 4 mM $MnCl_2$, 0.9 mM NAD^+, 50 mM DTT, or 1 mM EDTA under otherwise identical conditions (data not shown). These results suggest that AglA is active as a dimer of two identical 55-kDa subunits.

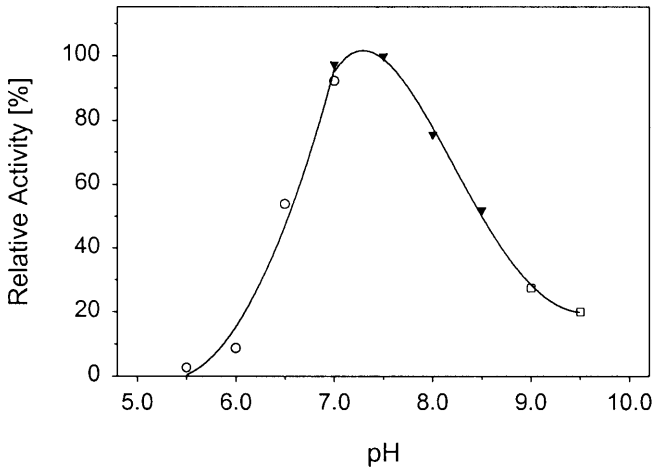
AglA displayed maximum activity at pH 7.5 and 90°C, with more than 50% relative activity at pH values between 6.5 and 8.5 and temperatures between about 65° and 100°C (Fig. 6). The residual activity of the *T. maritima* α-glucosidase after preincubation at temperatures between 50° and 90°C for various periods up to 2 days was followed. Surprisingly, limited inactivation was observed even at "low" temperatures (50° and 70°C), but in these cases the residual activity remained constant at about 50% (50°C) or dropped more slowly to about 20% of the initial activity (70°C) within 48 h (Fig. 6). The data indicate that heat inactivation during these long-term experiments is not first order. Apparently, the initial relatively rapid inactivation results in a more stable intermediate.

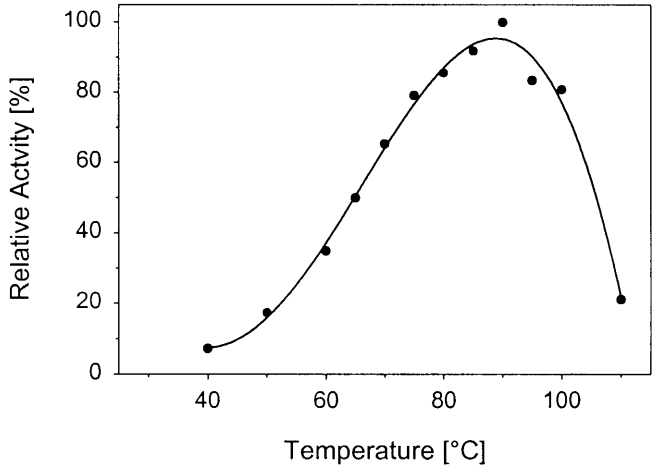
To assess the substrate specificity of AglA, the enzyme was challenged with numerous oligo- and polysaccharides as well as synthetic aryl and alkyl glycosides. Thin-layer chromatographic analysis (data not shown) revealed that none of the various polysaccharides tested (soluble starch, pullulan, glycogen, mannan, laminarin, chitin) were hydrolyzed by AglA. On the other hand, AglA was able to cleave various synthetic substrates and naturally occurring di- and trisaccharides, which are listed in Table 2. None of the available cyclic maltooligosaccharides α-, β-, or γ-cyclodextrin were attacked. The cleavage of *n*-dodecyl-β-D-maltoside or the chromogenic substrates pNP-α-D-glucoside, 4-*O*-methylumbelliferyl-α-D-glucoside, pNP-α-D-galactoside, and 4-*O*-methylumbelliferyl-α-D-galactoside indicated that bulky substituents on the aglycone side of the cleaved bond were no hindrance for glycosidic bond hydrolysis.

The fact that AglA liberated *p*-nitrophenol from pNP-α-D-glucoside and also from pNP-α-D-galactoside means that AglA is not specific with respect to the configuration at C-4 of the glycone moiety of its substrates. The relative activities measured with these substrates (determined at 10 mM substrate concentration at 60°C) were 100% and 114%, respectively. The results of kinetic data obtained with pNP-α-D-glucoside and pNP-α-D-galactoside at substrate concentrations between 0.01 and 50 mM are summarized in Table 3. The K_m values as derived from Eadie–Hofstee plots indicate a slightly higher affinity for the glucoside substrate.

Only α-glycosidic bonds were cleaved by AglA. In *n*-dodecyl-β-D-maltoside, only the α-glycosidic bond of the maltosyl moiety was cleaved, yielding glucose and *n*-dodecyl-β-D-glucoside. Disaccharides with β-glycosidic linkages such as cellobiose [*O*-β-D-glucopyranosyl-(1 → 4)-D-glucose] or lactose [*O*-β-D-galactopyranosyl-(1 → 4)-D-glucose] were not hydrolyzed.

The hydrolysis of maltooligosaccharides as the likely physiological substrates for AglA was investigated in more detail. The thin-layer chromatographic data gathered with linear maltooligosaccharides maltose (G2) through maltoheptaose (G7) as well as some chromogenic





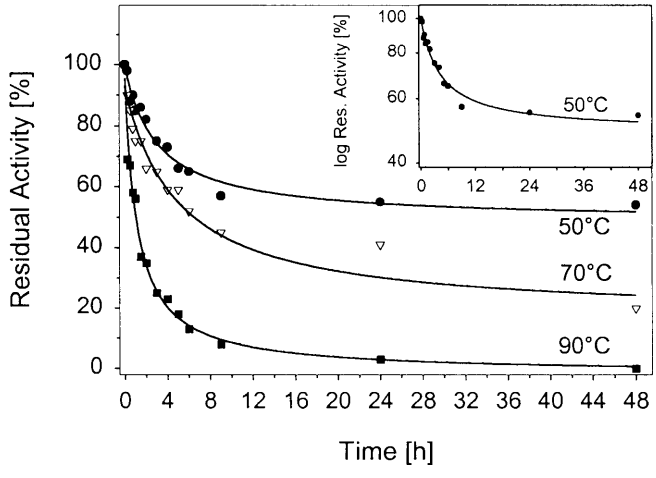


Fig. 6. pH and temperature dependence of activity and thermoinactivation kinetics of AglA. For determination of the pH dependence of AglA activity (*top graph*), assay mixtures containing all cofactors were adjusted to various pH values with 0.1 M sodium succinate buffer (pH 5.5–7.0), 0.1 M Tris-HCl (pH 7.0–8.5), or 0.1 M AMPSO buffer (pH 9.0–9.5) and incubated with enzyme (4.2 μg ml^{-1}) under otherwise standard conditions. The influence of temperature on enzyme activity (*middle graph*) was analyzed by incubating standard assay mixtures containing all cofactors with 5 μg ml^{-1} enzyme at various temperatures (40°–110°C) for 4 min. The influence of high temperatures on the kinetics of enzyme thermoinactivation was monitored by preincubation of purified α-glucosidase at a concentration of 0.22 mg ml^{-1} with 50 mM Tris-HCl pH 7.0 at temperatures between 50°C and 90°C. Aliquots (2.2 μg) of the enzyme were withdrawn periodically, and the residual activity was determined (*bottom graph*). The data collected at 50°C are also shown as a semilogarithmic plot (*inset in bottom graph*)

maltooligosaccharide substrates (pNP-G1 through pNP-G4) indicated that the cleavage efficiency decreased with increasing chain length (G2 > G3 > G4 > G5) (data not shown). Maltopentaose was an extremely poor substrate, whereas maltohexaose and maltoheptaose were not degraded significantly under our assay conditions.

Discussion

The α-glucosidase gene *aglA* and its genetic context

Comparison of *aglA* and surrounding sequences with sequence data of the *T. maritima* genome sequencing project available from the database of The Institute of Genome Research (TIGR, Rockville, MD, USA) revealed that the gene is located within a gene cluster involved in starch/maltodextrin utilization. *AglA* is one of several convergently oriented genes for maltodextrin transport and hydrolysis, in the order *malE-malF-malG-aglB-aglA*. This analysis also showed that two amylolytic genes described by us previously (Liebl et al. 1997), i.e., the genes for the maltodextrin-binding protein (*malE*) and the α-amylase gene (*amyA*), which is located upstream of but divergently oriented to *malE*, also belong to this gene cluster (see Fig. 1). The intergenic region between *malE* and *malF* contains a G/C-rich inverted repeat (5′-AAGAGGGGGACT TTTCCCCCTCTT-3′) that could be of importance to regulate the level of MalE production in relation to the level of MalF and MalG synthesis (for *E. coli*, it is known that the latter two are needed in stoichiometric amounts while the binding protein is produced in 100-fold excess) (Boos and Lucht 1996, Boos and Schuman 1998). The sequence data clearly indicate that the hyperthermophile *T. maritima* uses an *E. coli*-like periplasmic binding protein-dependent ABC transport system for maltodextrin uptake. Strikingly, the *T. maritima* genome (Nelson et al. 1999) contains two *malEFG* gene clusters in different regions of the chromosome. Because the one described here is embedded between amylolytic genes (*amyA*, *aglB*, *aglA*), it seems likely that it really plays a role in maltodextrin uptake. It is interesting in this context that the existence of a binding protein-dependent ABC transporter for maltose and trehalose was recently found in *Thermococcus (Tc.) litoralis*, a hyperthermophilic archaeon (Horlacher et al. 1998). Because organisms representing very deep phylogenetic branches in the domains of Archaea (*Tc. litoralis*) and Bacteria (*T. maritima*) possess binding protein-dependent maltodextrin uptake systems, it appears as though these high-affinity transport systems may have appeared very early in evolution. Remarkably, the gene order of the transport protein genes is the same in

Table 2. Substrate specificity of recombinant AglA, as determined by chromogenic enzyme assays and/or thin layer chromatography

Substrates	Nonsubstrates
pNP-α-D-Glucoside	α-, β-, γ-Cyclodextrin
Methyl-α-D-glucoside	Soluble starch
4-*O*-Methylumbelliferyl-α-D-glucoside	Pullulan
pNP-α-D-Galactoside	Glycogen
4-*O*-Methylumbelliferyl-α-D-galactoside	Mannan
n-Dodecyl-β-D-maltoside	Laminarin
Maltose [*O*-α-D-glucopyranosyl-(1 → 4)-D-glucose]	Chitin
Maltotriose	
Maltotetraose	
Isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucose]	
Panose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucopyranosyl-(1 → 4)-α-D-glucose]	
Trehalose [*O*-α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside]	
Melibiose [*O*-α-D-galactopyranosyl-(1 → 6)-D-glucose]	
Sucrose [*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside]	
Raffinose [*O*-α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside]	
Turanose [*O*-α-D-glucopyranosyl-(1 → 3)-β-D-fructofuranose]	
Melizitose [*O*-α-D-glucopyranosyl-(1 → 3)-*O*-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside]	

Table 3. Kinetic parameters of substrate hydrolysis by recombinant AglA, as determined under optimal reaction conditions

Substrate	v_{max} [μmol × min^{-1} × mg $protein^{-1}$]	K_m [mM]	k_{cat} [s^{-1}]
pNP-α-D-Glucoside	9.94	0.23	9.12
pNP-α-D-Galactoside	11.42	0.53	10.48

T. maritima as it is in *Tc. litoralis* and in the "modern" bacterium *E. coli*: *malE-malF-malG*.

Surprisingly, the amino acid sequence of the AglA of *T. maritima* was not related to other typical α-glucosidases that belong to the large α-amylase enzyme family [family 13 according to the classification of glycosyl hydrolases as proposed by Henrissat (1991)], but the enzyme shared low-level similarity (up to about 26% identity) with the melibiase MelA of *E. coli* and a putative enzyme of *B. subtilis*, as well as with a few bacterial 6-phospho-β-glucosidases (e.g., CelF proteins of *E. coli* and *B. subtilis*) and putative 6-phospho-β-glucosidases, and the recently identified 6-phospho-α-glucosidases of *Fusobacterium mortiferum* and *B. subtilis*, all of which belong to glycosyl hydrolase family 4. A second (putative) α-glucosidase gene, *aglB*, is present immediately upstream of *aglA*. At present, no data concerning the properties of AglB, which belongs to glycosyl hydrolase family 13, are available. It will be interesting to compare the properties, in particular the substrate profiles, of both α-glucosidases once AglB has been purified.

Substrate specificity of AglA

AglA is not specific with respect to the configuration at C-4 of the glycone (the sugar residue that contributes the anomeric carbon to the glycosidic bond) moiety of its substrates as α-glycosidic derivatives of D-galactose, the C-4 epimer of D-glucose, were also hydrolyzed (see Table 2). Also, AglA is not highly specific for α-1,4-glycosidic bonds like those in maltooligosaccharides, but also accepts alternate linkage types in other glucose- or galactose-based oligosaccharides, i.e., the α-1,6-bonds of isomaltose, melibiose, and raffinose, the α-1,1-bond of trehalose or the α-1,3-bonds of turanose and melizitose (see Table 2 for oligosaccharide structures). On the other hand, as is to be expected, AglA is very specific with respect to the anomeric configuration of the glycone residue, cleaving only α-glycosidic bonds but not β-glycosidic linkages.

The initial products of maltotetraose cleavage by AglA were glucose and maltotriose, not maltose (data not shown). Also, pNP-α-D-maltotetraoside hydrolysis yielded as initial products glucose + pNP-α-D-maltotrioside but not maltose + pNP-α-D-maltoside, maltotriose + pNP-α-D-glucoside, or maltotetraose + pNP. Similarly, the initial products of the cleavage of pNP-α-D-maltotrioside and pNP-α-D-maltoside were always glucose together with the pNP-substituted rest of the substrate molecule. The initial products of panose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucopyranosyl-(1 → 4)-α-D-glucose] hydrolysis were glucose and maltose, but no isomaltose was detected, indicating that the α-1,6-bond at the nonreducing end of this trisaccharide was cleaved first. Finally, none of the available cyclic maltooligosaccharides α-, β-, or γ-cyclodextrin were attacked. Taken together, these data show that AglA acts in an exo-fashion, liberating glucose (galactose) from the nonreducing end of its substrates. Although many exo-acting glycosyl hydrolases degrade their substrates from the nonreducing end, this is not a general rule. For example, the *E. coli* α-glucosidase MalZ releases glucose from the reducing end of maltotriose and longer maltooligosaccharides (Tapio et al. 1991). Another argument supporting that the nonreducing end-glucose/galactose is recognized and the adjacent α-glycosidic bond is cleaved by AglA comes from the broad acceptance of various differently α-linked glucose disaccharides (see foregoing). It would be difficult to

understand how α-1,4-, -1,6-, and -1,1-bonds could be cleaved if cleavage took place from the reducing end.

Judging from the in vitro substrate cleavage studies, AglA could be called an α-glycosidase with very broad specificity. The enzyme theoretically could be involved in the breakdown of various α-glucoside and/or α-galactoside substrates. However, based on the genetic context of the *aglA* gene (clustered with other amylolytic genes, encoding an α-amylase, maltodextrin uptake proteins, and a second α-glucosidase; see Fig. 1), it seems clear that the in vivo physiological role of this enzyme in *T. maritima* is to function as a true α-glucosidase in the course of starch and maltodextrin utilization. Like other typical α-glucosidases, AglA preferentially cleaves maltose and short malto-oligosaccharides, its activity decreasing markedly with increasing chain length of the substrate.

Cofactor requirement of AglA and other family 4 glycosyl hydrolases

Our initial attempts to purify the *T. maritima* α-glucosidase were hampered by a dramatic loss of enzyme activity during chromatographic purification steps and inactivation during storage. We found that the activity of the enzyme present in chromatographic fractions could be restored by the addition of a small amount of *E. coli* crude extract, indicating that the activating substance(s) were not *Thermotoga* specific. The reactivating compound(s) were able to pass through an ultrafiltration membrane with a cutoff limit of 3000 Da. Further complementation experiments with various chemically defined compounds and combinations thereof finally showed that this enzyme requires NAD^+ and Mn^{2+} for full activity, which had not been previously reported for an α-glucosidase. Furthermore, activity was only observed under reducing reaction conditions brought about by the addition of dithiothreitol, mercaptoethanol, or, less efficiently, cysteine. After the nucleotide sequence of the gene became available, it was found that AglA of *T. maritima* belongs to family 4 of glycosyl hydrolases. During the completion of this work, results demonstrating the NAD^+/NADH and metal ion dependence of GlvA, a phospho-α-glucosidase of *B. subtilis*, were reported (Thompson et al. 1998). Like AglA, GlvA belongs to family 4 of glycosyl hydrolases. Another family 4 enzyme, i.e., the α-galactosidase MelA of *E. coli*, was reported long ago to require NAD^+ and Mn^{2+} (Burstein and Kepes 1971), and other members of this family, i.e., the 6-phospho-α-glucosidase MalH of *Fusobacterium mortiferum* and the 6-phospho-β-glucosidase CelF of *E. coli* also are activated by these cofactors (Thompson et al. 1998, 1999; Bouma et al. 1997). Thus, it seems that the requirement of Mn^{2+} and NAD^+ are common features of the enzymes belonging to glycosyl hydrolase family 4. All known family 4 enzymes, including the *T. maritima* enzyme, are thought to be intracellular, which is in agreement with the unusual cofactor requirement. AglA is by far the most thermostable of the known NAD^+-, metal-requiring glycosyl hydrolases of family 4.

In this context it is of interest to compare *T. maritima* AglA with the recently described NAD^+- and metal-dependent phospho-α-glucosidase GlvA of *B. subtilis* (Thompson et al. 1998). The enzymes have completely different substrate profiles. While GlvA appears to be highly specific for glucoside substrates phosphorylated at C-6 of the glycone moiety (maltose 6-phosphate, trehalose 6-phosphate) and does not accept nonphosphorylated substrates, AglA has a very broad cleavage specificity and is able to hydrolyze a range of nonphosphorylated α-glucosides and α-galactosides with various linkage types (1,1-, 1,3-, 1,4-, 1,6-) and with various sugar and nonsugar aglycone moieties. Another striking difference between GlvA and AglA is the apparent oligomerization state of the native enzymes (see following).

Although calcium dependence is well documented for certain amylolytic enzymes, e.g., α-amylases (Vihinen and Mäntsälä 1989), manganese requirement is unusual for amylolytic enzymes and for glycosyl hydrolases in general. Most known α-glucosidases do not have an essential need of metal cations for activity. On the contrary, moderate to severe inhibition by Mn^{2+} and Co^{2+} has been reported for some α-glucosidases, i.e., the *Thermococcus* species strain AN1 enzyme and some *Bacillus* enzymes (Suzuki et al. 1992; Piller et al. 1996; Krohn and Lindsay 1991). The precise role for Mn^{2+} for enzyme activity of the glycosyl hydrolase family 4 enzymes is uncertain. Thompson et al. (1998) reported that in its metal-free form *B. subtilis* GlvA exists as an inactive dimer, which in the presence of Mn^{2+} associates to the catalytically active tetramer. On the other hand, with *T. maritima* AglA we observed the same oligomerization state (dimer) for metal-depleted enzyme (no metal addition or treatment with 1 mM EDTA) as well as for enzyme supplemented with 1 mM or 4 mM $MnCl_2$. Therefore, the control of subunit association does not seem to be a role for Mn^{2+} here. The other cofactors NAD^+ (0.9 mM) or DTT (50 mM) also had no effect on the apparent oligomerization state of AglA, as was determined with gel permeation chromatography. In the case of AglA, Mn^{2+} could be important for intrasubunit conformation, for substrate binding, or be directly involved in catalysis. A plausible candidate for involvement in metal coordination is C-174 of AglA. This highly conserved cysteine is present at corresponding positions in all known members of glycosyl hydrolase family 4.

The common features of family 4 glycosyl hydrolases, as investigated so far, may be summarized as follows: (i) relative instability of the enzymes; (ii) requirement of Mn^{2+} (or Co^{2+}, Ni^{2+}, or Fe^{2+}) for activity; (iii) requirement of NAD^+ for activity; (iv) oligomeric quarternary structure (homodimer or -tetramer); (v) subunit size about 51 kDa (about 450 residues); and (vi) glycone specificity for glucose, its C-4 epimer galactose or both, or for glucose-6-phosphate (Thompson et al. 1998; Burstein and Kepes 1971; Bouma et al. 1997; Nagao et al. 1988; this work). Taking these features as a measure, family 4 appears to be a quite coherent group of biochemically and structurally similar enzymes. It is interesting to note that no eukaryal or archaeal members of this unique enzyme type have yet been found.

The physiological rationale of activation by NAD^+ of *T. maritima* AglA and other family 4 enzymes that are glycosyl hydrolyses rather than oxidoreductases remains unclear. However, it may be possible that the NAD^+/NADH ratio is sensed by the enzyme. This sensitivity could be useful to regulate the flow of carbon into the glycolytic pathway. In fermentative metabolism, as found in the strict anaerobe *T. maritima*, it is useless to feed further glucose into glycolysis when NAD^+ becomes limiting. In respiratory metabolism, where NADH is regarded to be one of the central signals for energy availability, it may make sense that the hydrolysis of oligosaccharides is stimulated under conditions of energy depletion (increased NAD^+ level). Unfortunately, this possible explanation is not fully satisfactory for (at least) two reasons. First, the NAD^+ concentration in the facultative anaerobe, *E. coli*, is about 0.8 mM, but strict anaerobes are believed to contain a significantly higher concentration of pyridine nucleotides (about two- to fourfold; see London and Knight 1966; Penfound and Foster 1996). However, NAD^+ concentrations of 0.9 mM or more represent saturating levels for AglA activation (but it should be kept in mind that the in vivo levels of NAD/NADH in *T. maritima* have never been measured). Second, it is important to remember that NAD^+ activation is the exception in glycosyl hydrolases and has been found only in family 4 enzymes whereas the majority of glycosyl hydrolases, including many other intracellular oligosaccharidases, are not NAD^+ activated. Thus, the reason for NAD^+-mediated activation remains obscure.

The discovery of a NAD^+-activated glycosyl hydrolase in *Thermotoga*, a very deep branch in the phylogenetic tree, makes it interesting to take into consideration also the aspect of evolution. This finding could be an indication that NAD^+-activated oligosaccharidases appeared relatively early in bacterial evolution and that the representatives found now may be a relict of an ancient pathway of saccharolytic metabolism possibly regulated at the level of oligosaccharide hydrolysis. Clearly, more work on the regulation of the activity and biosynthesis of these enzymes is needed to clarify their role in bacterial metabolism.

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