A Novel Branching Enzyme of the GH-57 Family in the Hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1

Taira Murakami,¹ Tamotsu Kanai,¹ Hiroki Takata,² Takashi Kuriki,² and Tadayuki Imanaka^{1*}

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan,¹ and Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Nishiyodogawa-ku, Osaka, 555-8502, Japan²

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Branching enzyme (BE) catalyzes formation of the branch points in glycogen and amylopectin by cleavage of the α -1,4 linkage and its subsequent transfer to the α -1,6 position. We have identified a novel BE encoded by an uncharacterized open reading frame (TK1436) of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. TK1436 encodes a conserved protein showing similarity to members of glycoside hydrolase family 57 (GH-57 family). At the C terminus of the TK1436 protein, two copies of a helix-hairpin-helix (HhH) motif were found. TK1436 orthologs are distributed in archaea of the order *Thermococcales*, cyanobacteria, some actinobacteria, and a few other bacterial species. When recombinant TK1436 protein was incubated with amylose used as the substrate, a product peak was detected by high-performance anion-exchange chromatography, eluting more slowly than the substrate. Isoamylase treatment of the reaction mixture significantly increased the level of short-chain α -glucans, indicating that the reaction product contained many α -1,6 branching points. The TK1436 protein showed an optimal pH of 7.0, an optimal temperature of 70°C, and thermostability up to 90°C, as determined by the iodine-staining assay. These properties were the same when a protein devoid of HhH motifs (the TK1436 Δ H protein) was used. The average molecular weight of branched glucan after reaction with the TK1436 Δ H protein was over 100 times larger than that of the starting substrate. These results clearly indicate that TK1436 encodes a structurally novel BE belonging to the GH-57 family. Identification of an overlooked BE species provides new insights into glycogen biosynthesis in microorganisms.

Glycogen and starch are the two major storage materials in living organisms (3). Both are polysaccharides of an α -1,4-linked glucose polymer with occasional branch points using α -1,6-glucosidic bonds, although the branches of glycogen tend to be shorter (8 to 10 glucose units) than those of starch amylopectin (12 to 20 glucose units). These branched structures are important in enhancing catabolic reaction rates by increasing reactive termini and also serve to increase polymer solubilities. The biosynthetic pathways for the two materials are similar. Both are synthesized from glucose 1-phosphate by the following three reaction steps: (i) synthesis of ADP (or UDP)-glucose, (ii) elongation of α -1,4-linked chains, and (iii) branch chain formation through α -1,6-glucosidic bonds (3, 9).

Branching enzyme (BE) [1,4- α -D-glucan:1,4- α -D-glucan 6- α -D-(1,4- α -D-glucano)-transferase] (EC 2.4.1.18) catalyzes the formation of branch points in glycogen and amylopectin by cleavage of α -1,4-glucosidic bonds and its subsequent transfer to α -1,6 positions. BEs identified to date all belong to family 13 of the glycoside hydrolases (the GH-13 family) according to Henrissat's classification (12–14). The GH-13 family is a large enzyme family that constitutes about 20 different reactions and product specificities. This family includes most of the amylo-

lytic enzymes showing preferences for hydrolysis and transglycosylation of α -1,4- and α -1,6-glycosidic bonds (<http://www.cazy.org/CAZY/>).

The “ α -amylase family,” an enzyme family proposed by Kuriki and Imanaka and by Takata et al. in 1992 and thereafter (25, 37), includes many proteins in the GH-13 family, such as α -amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41), isoamylase (EC 3.2.1.68), neopullulanase (EC 3.2.1.135), cyclodextrin glucanotransferase (EC 2.4.1.19), and BE. Proteins in the α -amylase family have four conserved sequence motifs where catalytic residues and most other residues involved in substrate binding are located (25). BE also contains the four sequence motifs, and amino acid residues located in these regions (such as Asp⁴⁰⁵, Glu⁴⁵⁸, and Asp⁵²⁶ in the *Escherichia coli* BE numbering) have been shown, by site-specific mutagenesis, to be important for BE activity (24, 39). The three-dimensional structure of BE (1) supports this view.

In this paper, we identify a novel BE in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. The new BE belongs to family 57 of glycoside hydrolases (the GH-57 family) and is structurally distinct from the previously characterized BEs of the GH-13/ α -amylase family.

MATERIALS AND METHODS

Bioinformatics. Sequences showing similarity to the TK1436 protein were found by NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the collected sequences were aligned with the CLUSTAL W program at the DNA Data Bank of Japan website (<http://www.ddbj.nig.ac.jp/search/clustalw-e.html>). To search for specific motifs in protein sequences, a GenomeNet server (<http://motif.genome.jp/>) was used.

* Corresponding author. Mailing address: Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan. Phone: 81-75-383-2777. Fax: 81-75-383-2778. E-mail: imanaka@sbchem.kyoto-u.ac.jp.

Microorganisms, plasmids, and medium. *Escherichia coli* DH5 α and plasmid pUC118 were used for general DNA manipulation and sequencing. *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA) and pET21a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (10 g liter⁻¹ of tryptone, 5 g liter⁻¹ of yeast extract, and 10 g liter⁻¹ of NaCl) at 37°C. Ampicillin was added to the medium at a concentration of 100 μ g ml⁻¹.

To prepare RNA for microarray analysis, *T. kodakaraensis* KOD1 (2, 28) was grown in MA-YT medium (20) containing artificial sea salts, yeast extract, and tryptone. When growth with elemental sulfur was required, sulfur powder (Wako Pure Chemical Industries, Osaka, Japan) was added at a concentration of 5 g liter⁻¹ after the MA-YT medium had been autoclaved. For cultivation with maltodextrin, 5 g liter⁻¹ Amycol no. 3-L (Nippon Starch Chemical, Osaka, Japan) was added to the MA-YT medium before it was autoclaved. Cultivation was performed anaerobically at 85°C, and cells in early log phase were harvested.

DNA manipulations and sequencing. DNA manipulations were performed by standard protocols as described by Sambrook and Russell (32). Restriction and modification enzymes were purchased from Toyobo (Osaka, Japan) or Takara Bio (Otsu, Japan). Small-scale preparations of plasmid DNA were performed with the QIAGEN plasmid mini kit (QIAGEN, Hilden, Germany). KOD Plus (Toyobo) was used as the DNA polymerase for PCR. DNA fragments after agarose gel electrophoresis were recovered with the GFX PCR DNA and a Gel Band Purification Kit (Amersham Bioscience, Little Chalfont, United Kingdom). DNA sequencing was performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit and a Model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Recombinant-protein production in *E. coli*. Recombinant TK1436 protein (amino acids [aa] 1 to 675) and a deletion derivative devoid of the C-terminal two-copy helix-hairpin-helix (HhH)₂ motif (TK1436 Δ H; aa 1 to 562) were prepared as follows. With *T. kodakaraensis* genomic DNA as a template, the following two oligonucleotide primers were used to amplify the gene encoding the TK1436 protein. The sense primer was 5'-GTGATCATATGAAGGGTTACCTGACTTTTG-3', and the antisense primer was 5'-AAGGGATCCTCACTCCACTTGAGCTATGAAC-3'. The underlined sequences indicate NdeI and BamHI sites in the sense and antisense primers, respectively. To amplify the gene encoding the TK1436 Δ H protein, the same procedure was used, except that the antisense primer 5'-GTTGGGATCCCTCAGAGAACCTTGGGCTTTTCCTC-3' was employed. The underlined sequence indicates a BamHI site. These amplified fragments were inserted into pUC118. After confirming the absence of unintended mutations in the amplified regions, NdeI-BamHI restriction fragments were inserted into pET21a(+) at the appropriate sites, and the resulting plasmids were named p1436WT (expressing the TK1436 protein) and p1436DH (expressing the TK1436 Δ H protein). *E. coli* BL21-CodonPlus(DE3)-RIL cells harboring these plasmids were grown in LB medium at 37°C, and gene expressions were induced by the addition of 0.1 mM isopropyl- β -D-thiogalactopyranoside in the exponential phase of growth. Following further cultivation for 5 h, the cells were harvested by centrifugation (4,500 $\times$ g; 4°C; 15 min) and resuspended in 50 mM Tris-HCl buffer (pH 8.0). After a further round of centrifugation, the cell pellets were stored at -80°C until they were used.

Purification of recombinant TK1436 and TK1436 Δ H proteins. *E. coli* cells carrying p1436WT were suspended in 50 mM Tris-HCl buffer (pH 8.0) supplemented with protease inhibitors (Complete Mini; Roche Diagnostics, Basel, Switzerland) and disrupted using a sonicator (UD-201; TOMY, Tokyo, Japan). The cell lysate was centrifuged (20,400 $\times$ g; 4°C; 30 min), and the supernatant was incubated at 80°C for 10 min to remove heat-labile proteins derived from host cells. The protein fraction after heat treatment was applied to an anion-exchange column, RESOURCE Q (6 ml; Amersham Biosciences), and the TK1436 protein was eluted with 50 mM Tris-HCl buffer (pH 8.0) at a salt concentration of 0.1 to 0.3 M NaCl. Protein fractions were collected and applied to a hydrophobic column, RESOURCE PHE (6 ml; Amersham Biosciences). TK1436 protein was eluted with 50 mM sodium phosphate (pH 7.5) at a salt concentration of ca. 0.6 M (NH₄)₂SO₄. The eluted fraction was dialyzed against 10 mM sodium phosphate buffer (pH 7.5) using the Slide-A-Lyzer Dialysis Cassette (Pierce, Erembodegem, Belgium). The protein concentration was determined with the Protein Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions, with bovine serum albumin as the standard. For purification of the TK1436 Δ H protein, the above-mentioned procedure was used, except that *E. coli* cells carrying p1436DH were employed.

Enzyme assay using TLC. Activity assays for amylolytic enzymes were performed using the following saccharides: melibiose (Wako Pure Chemical Industries), pullulan (Nacalai Tesque, Kyoto, Japan), maltoheptaose (Hayashibara Biochemical Laboratories, Okayama, Japan), and amylose AS-30 (Nakano Vinegar, Handa, Japan). Amylose AS-30 is an enzymatically synthesized amylose having an

average M_w of 3.0×10^4 . A reaction mixture (25 μ l) containing 0.1 mg substrate, 8% (vol/vol) dimethyl sulfoxide (DMSO), and 0.01% (vol/vol) TritonX-100 in 10 mM sodium phosphate buffer (pH 7.5) was incubated for 2 h at 70°C with or without the addition of 1 μ g enzyme. To detect branching-enzyme activity, isoamylase digestion was performed on the amylose-containing reaction mixture. For 25 μ l of reaction mixture, 0.4 μ l of 1 M HCl, 0.5 μ l of 1 M sodium acetate buffer (pH 3.5), and 1.25 μ l of 0.1 mg ml⁻¹ isoamylase from *Pseudomonas amyloclavata* ATCC 21262 (Hayashibara) were added, and incubation was continued overnight at 37°C. For product detection by thin-layer chromatography (TLC), aliquots (10 μ l) of reaction mixtures were chromatographed on a silica gel plate (Kieselgel 60; Merck, Berlin, Germany) with isopropyl alcohol-acetone-water (2:2:1 [vol/vol/vol]), and saccharides were detected by spraying the plate with aniline-diphenylamine reagent (4 ml aniline, 4 g diphenylamine, 200 ml acetone, and 30 ml 85% [vol/vol] phosphoric acid).

Analysis of chain length distribution. The chain length distribution of glucan was analyzed by high-performance anion-exchange chromatography (HPAEC) with a pulsed amperometric detector (Dionex, CA), as described by Takata et al. (41). To avoid precipitation of glucans, 5 μ l of 1 M sodium hydroxide solution was added to a reaction mixture of 25 μ l, and sample solution was filtered through a 0.45- μ m-pore-size membrane before injection.

Quantitative BE assay. To assay BE quantitatively, an iodine-staining assay was performed as described by Takata et al. (38) with slight modifications. The stock enzyme solution was first diluted to 0.1 mg ml⁻¹ with 10 mM sodium phosphate buffer (pH 7.5) containing 0.05% (vol/vol) Triton X-100, and this solution was further diluted to an appropriate concentration (0.001 to 0.01 mg ml⁻¹) with distilled water. A substrate solution was prepared by mixing 100 μ l of 1.2% (wt/vol) type III amylose (Sigma, St. Louis, MO) dissolved in DMSO, 200 μ l of 200 mM sodium phosphate buffer (pH 6.5), and 700 μ l of distilled water. The diluted enzyme solution (40 μ l) was mixed with the same volume of the substrate solution and incubated at 60°C or 70°C for 10 min. After incubation, 400 μ l of 0.4 M HCl was added to stop the reaction, and 400 μ l of iodine reagent was then added to the solution. The iodine reagent was made daily from 0.125 ml of stock solution (0.26 g I₂ and 2.6 g KI in 10 ml water), 0.5 ml of 1 M HCl, and deionized water to make a final volume of 65 ml. BE activity was determined by measuring the decrease in absorbance of this solution at 660 nm.

Determination of glucan molecular weights. The molecular weights of glucans were determined as follows. A reaction mixture (500 μ l) containing 2.0 mg of amylose AS-30, 8% (vol/vol) DMSO, 0.01% (vol/vol) Triton X-100, and 20 μ g of BE protein (the TK1436 Δ H protein) in 10 mM sodium phosphate buffer (pH 7.5), was incubated for 2 h at 70°C. After incubation, HCl was added to a final concentration of 16 mM, and the mixture was further incubated for 10 min at 100°C. Glucans in the reaction mixture were analyzed by high-performance liquid chromatography (HPLC) using a multiangle laser-light-scattering photometer (DAWN DSP; Wyatt Technology, Santa Barbara, CA) and a differential refractive-index detector (RI-71; Showa Denko, Tokyo, Japan), as described by Takata et al. (38).

β -Amylase treatment. To a 25- μ l reaction mixture after isoamylase digestion, 5 μ l of 1 M sodium acetate (pH 6.0) and 1 μ l of β -amylase crystalline suspension from sweet potato (Sigma) were added, and the mixture was incubated at 37°C for 3 h. The reaction was stopped by boiling the mixture at 100°C for 5 min, and the mixture was analyzed by HPAEC.

Estimation of the molecular mass of the native protein. The molecular mass of the native protein was calculated by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences) with a mobile phase of 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. The void volume was determined using blue dextran, and a standard calibration curve was obtained using RNase A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), albumin (67.0 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

Protein 3D structure generation by homology modeling. A three-dimensional (3D) structural model of the TK1436 protein was generated using a comparative-modeling technique. Based on the crystal structure of TT1467 (now renamed TTHA1902) from *Thermus thermophilus* HB8 (Protein Data Bank accession number 1UFA), a model structure of the TK1436 protein was generated using the SWISS-MODEL Protein Modeling Server (<http://swissmodel.expasy.org//SWISS-MODEL.html>) (33). The structure obtained was visualized using the PyMOL program for MacOSX (<http://pymol.sourceforge.net/>).

RESULTS

Identification of TK1436 as a maltodextrin-induced open reading frame (ORF). *T. kodakaraensis* KOD1 is a hyperther-

mophilic archaeon belonging to the order *Thermococcales*. It is an anaerobic heterotroph that utilizes peptide-related substrates in the presence of elemental sulfur (S^0) and also utilizes starch without a requirement for S^0 (2, 28). Consideration of genome information (10) and biochemical characteristics of the strain and related species led to the proposal of a glycolytic pathway relevant to starch metabolism (20).

Using a DNA microarray that covers 96.5% of *T. kodakaraensis* ORFs, we performed a transcriptome analysis to exhaustively identify genes related to α -glucan metabolism in the strain (unpublished data). A maltodextrin (Ameycol no. 3-L) containing maltooligosaccharides with a degree of polymerization (DP) of 1 to 12 was used as the source of α -glucan. (For more detailed information on cell cultivation, see Materials and Methods.) Among genes whose transcription was induced by the addition of maltodextrin, an ORF encoding an unknown protein (TK1436) was identified. The signal intensity of TK1436 was induced 3.2-fold under glycolytic conditions (MA-YT medium with maltodextrin) compared to the level seen under peptide-utilizing conditions (MA-YT medium with S^0).

Primary structure of the TK1436 protein. TK1436 encodes a protein of 675 aa with a calculated molecular mass of 78,545 Da. It is an uncharacterized conserved protein annotated as a "probable α -amylase" by the *T. kodakaraensis* genome project (10). According to the NCBI Conserved Domain Database, TK1436 is assigned to the COG1543 group. The functions of the group members are unknown.

A long N-terminal region (1 to 534 aa) of the TK1436 protein shows similarity to amino acid stretches of proteins in the GH-57 family. Phylogenetic analysis of family members indicated that the GH-57 family is divided into seven subfamilies and three independent members (46). Four subfamilies include proteins of characterized specificities (α -amylase, 4- α -glucanotransferase, amylopullulanase, and α -galactosidase). The TK1436 protein belongs to a subfamily in which no protein has been characterized. Multiple alignments of subfamily members showed the existence of six conserved sequence regions (regions A to F in Table 1). Regions A, B, C, E, and F essentially correspond to the five conserved sequence motifs in the GH-57 family (46). Two catalytic residues of the GH-57 family, initially identified in a series of biochemical and structural studies of *Thermococcus litoralis* 4- α -glucanotransferase (16, 17) and later shown to be conserved in other family members (18, 46), are located in region C (Glu¹⁸³, a nucleophile) and region E (Asp³⁵⁴, an acid/base catalyst). On the other hand, region D is a region conserved only in the TK1436 subfamily. At the C terminus of the TK1436 protein (aa 620 to 675), two copies of an HhH motif exist. The HhH motif is a domain of around 20 amino acids and is generally regarded as a sequence-nonspecific DNA binding motif (34).

Determination of the enzymatic activity of the TK1436 protein. In order to elucidate the function of the TK1436 protein, recombinant protein was prepared using *E. coli* cells as hosts. In addition to the full-length protein (the TK1436 protein; 675 aa), a recombinant protein devoid of both copies of HhH motifs (the TK1436 Δ H protein; 562 aa) was prepared. Although the two recombinant proteins were produced and purified by the same methods, the protein yield of the full-length protein was much lower (10% or less) than that of the

TABLE 1. Conserved sequence motifs among TK1436 orthologs^a

Locus name	Region A	Region B	Region C	Region D	Region E	Region F
TK1436	7_FVLHHTL PYV	134_GYVEV I	178_GIWL PRCAY	269_VMSAHYGYPGDFW YREFH	353_YDT ELF GHWF EG	438_RELLILISA SDWQFLI
PF1393	7_FVLHSHI PYV	136_GYLET I	180_GMML PRCAY	271_VMSADIGYPGDFW YREFH	355_YDT ELF GHWF EG	460_RELLILISA SDWQFLI
TT141902	6_LVLHSHI PYV	135_GQVEL I	179_GFML PRCAY	273_VMSADYGYPGDFW YREFH	352_YDA ELF GHWF EG	451_RELLILISA SDWQFLI
sl0735	8_LVLHSHI PFV	139_NNLET I	184_GIWL PRCAY	249_VMSQVGYPGDFW YREFY	352_YDA ELF GHWF EG	457_RELLILISA SDWQFLI
CAC2414	8_FVLHSHI PFV	139_GCVET I	184_GIWL PRCAY	249_VMSQVGYPGDFW YREFY	349_YDT ELF GHWF EG	455_RELLILISA SDWQFLI
Rv3031	14_LVLHSHI PNL	157_GTVET I	200_GIWL PRCAY	259_VMSQVGYPGDFW YREFY	343_FDT ELF GHWF EG	453_RELLILISA SDWQFLI
Consensus	*** *	*	* ** *	*** ** *	* ** *	*** *

^a TK1436 orthologous proteins shown in this table are from *Thermococcus kodakaraensis* KOD1 (TK1436; accession no. BAD85625), *Pyrococcus furiosus* DSM 3638 (PF1393; accession no. AAL81517), *Thermophilus* HB8 (TT141902; accession no. YP_145168), *Synechocystis* sp. strain PCC 6803 (sl0735; accession no. NP_442931), *Clostridium acetobutylicum* ATCC 824 (CAC2414; accession no. AAK80369), and *Mycobacterium tuberculosis* H37Rv (Rv3031; accession no. NP_217547). Italic letters and asterisks below the sequence represent the identical amino acids among the six sequences compared. The two GH-57 catalytic residues, Glu¹⁸³ and Asp³⁵⁴ (in TK1436 protein numbering), are highlighted in boldface.

FIG. 1. Reaction specificity of the TK1436 protein. Shown are thin-layer chromatograms of reaction mixtures containing various saccharides used as enzyme substrates. The saccharides used were melibiose (MEL), pullulan (PUL), maltoheptaose (G7), and amylose AS-30 (AMY). Reaction mixtures were incubated at 70°C with (+) or without (–) the TK1436 protein. In the case of the reaction mixture containing amylose, isoamylase treatment (++) was performed after reaction with the TK1436 protein. Lane M is a standard maltooligosaccharide mixture containing glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltohexaose (G6).

TK1436 Δ H protein because of low production yield in the host cells and instability during purification.

Using the full-length protein, we first examined whether the TK1436 protein showed any enzyme activities known in the GH-57 family. According to the literature, four enzyme activities have been detected in the family (18, 46). The activities are α -amylase (22, 27), 4- α -glucanotransferase (19, 26, 29, 35), amylopullulanase (5, 7, 8), and α -galactosidase (43). The TK1436 protein was incubated at 70°C with melibiose, pullulan, maltoheptaose, or amylose as the substrate, and the reaction mixtures were analyzed by TLC (Fig. 1). No mobility change was observed in reaction mixtures containing melibiose or pullulan, showing that the TK1436 protein contained neither α -galactosidase nor pullulanase activity. Similarly, hydrolysis or transglycosylation of maltoheptaose could not be observed, suggesting that the TK1436 protein contained neither α -amylase nor 4- α -glucanotransferase activity. However, in the case of amylose, small but significant amounts of short-chain glucans were detected.

The distinct specificities of the TK1436 protein for two α -glucans (maltoheptaose and amylose) are seemingly conflicting. However, BE is reported to produce short-chain α -1,4-glucan as a side product when amylose is used as a substrate (39, 41). We next asked, therefore, if the TK1436 protein showed BE specificity. The reaction mixture after incubation with the TK1436 protein was further treated with isoamylase, catalyzing hydrolysis of α -1,6 linkages. As a result, the levels of maltooligosaccharides increased greatly, suggesting that the reaction product contained many α -1,6 branch points. Next, the

reaction mixtures were analyzed in detail by HPAEC (Fig. 2). The mixture after reaction with the TK1436 protein contained, in addition to short-chain α -1,4-glucans, a peak corresponding to a high-molecular-weight product eluting at around 43 min (Fig. 2A, chromatogram 2). Isoamylase treatment of the reaction mixture significantly increased the amounts of short-chain α -1,4-glucans with concomitant disappearance of the high-molecular-weight product (Fig. 2A, chromatogram 3). The amylose used in this experiment (amylose AS-30) contains negligible levels of α -1,6 branch points, as isoamylase treatment of the substrate did not change the chromatographic profile (data not shown). In summary, these results clearly indicate that the TK1436 protein contains BE activity that was not previously identified in the GH-57 family of enzymes.

The chain length distribution of the reaction products showed DPs of 5 to 30, with two local maxima at DP 6 and DP 11. The branch length distribution is much different from that obtained with *Aquifex aeolicus* BE, products of which showed a branch chain distribution of DP 5 to 40, with a sharp peak at DP 10 (38). The reaction product of the TK1436 Δ H protein showed basically the same properties as that of the TK1436 protein (Fig. 2A, chromatograms 4 and 5).

Enzymatic characterization of recombinant TK1436 and TK1436 Δ H proteins. The optimal pH activity values, optimal temperature activity values, and thermostabilities of the TK1436 and TK1436 Δ H proteins were determined by the iodine-staining assay. The two proteins showed the same optimal pH value of 7.0, functioned optimally at a temperature of 70°C, and had the same specific activities (the TK1436 Δ H protein

FIG. 2. (A) HPAEC analysis. (1) The amylose AS-30 substrate, (2) products after treatment of amylose AS-30 with TK1436 protein, (3) isoamylase-digested products of step 2, (4) products after treatment of amylose AS-30 with TK1436ΔH protein, and (5) isoamylase-digested products of step 4. Peaks eluting at 40 to 45 min in the chromatograms are the substrate (41.4 min; shown as S) and branched products (43.3 min; shown as P). The numbers on the peaks indicate chain lengths. (B) Schematic description of the branching and debranching of α-glucan by BE and isoamylase. The circles, bars, and arrows stand for glucosyl residues, the α-1,4-glucosidic linkages, and the α-1,6-glucosidic linkages, respectively.

had $95.6 \pm 4.6\%$ of the activity of the TK1436 protein). Both proteins were stable up to 90°C , and activities decreased gradually during incubation at 100°C . The thermostability was even higher than that of *A. aeolicus* BE, which is the most thermostable BE previously reported, with stability at temperatures up to 85°C (38). In Fig. 3, the data for the TK1436ΔH protein are shown as a representative.

Detailed characterization of the reaction product and enzyme. Although no significant differences in enzymatic characteristics existed between the TK1436 and TK1436ΔH proteins, there was a big difference in the storage stabilities of the proteins. The TK1436 protein showed rapid degradation within 2 to 3 days of storage at 4°C , while the TK1436ΔH protein was stable under the same conditions (data not shown).

Because of this, further studies of the enzyme and product characteristics were performed using the TK1436ΔH protein.

The molecular weights of the reaction products were determined by HPLC–multiangle laser-light-scattering–refractive-index analysis. Amylose AS-30 was used as a substrate, and a time course of reaction products was analyzed (Fig. 4). A sharp peak at zero hours of incubation corresponded to the substrate (peak 2), with an average M_w of 4.5×10^4 . As the reaction proceeded, the substrate peak gradually disappeared, and two other peaks were generated. A peak appearing faster than peak 2 (peak 1) corresponded to branched products with an average M_w of 3.0×10^6 to 3.5×10^6 . The value was about 100 times greater than that of the starting substrate. On the other hand, the other peak, appearing more slowly than peak 2 (peak

FIG. 3. (A) Optimal pH of TK1436ΔH protein. Enzyme activities were assayed at 60°C. The buffers used were as follows: sodium citrate buffer (◆) (pH 3.6, 4.2, and 4.7), sodium acetate buffer (■) (pH 4.1, 4.6, 5.1, and 5.6), MES (morpholineethanesulfonic acid) buffer (▲) (pH 5.6, 6.0, 6.5, and 7.1), sodium phosphate buffer (◇) (pH 7.0, 7.4, 7.9, and 8.2), Tris-HCl buffer (X) (pH 7.7, 8.2, 8.7, and 9.1), and CHES (2-cyclohexylaminoethanesulfonic acid) buffer (●) (pH 9.0, 9.5, and 9.9). (B) Optimal temperature of TK1436ΔH protein. Enzyme activities were assayed at pH 7.0. (C) Thermostability of TK1436ΔH protein. Enzymes in 10 mM sodium phosphate buffer (pH 7.5) were incubated at 80°C (◆), 90°C (■), or 100°C (▲) for the times shown and then immediately cooled on ice. Residual activities were assayed at 60°C and pH 7.0.

3), corresponded to short-chain glucans with an average M_w of 4.8×10^3 to 2.0×10^4 .

To confirm that the branched products did not contain linkages other than α -1,4- and α -1,6-glucosidic bonds, reaction products after isoamylase digestion were treated with β -amylase and analyzed by HPAEC. No glucan except maltose and glucose was detected (data not shown), indicating that branched products contained only α -1,4 and α -1,6 linkages, similar to glucans made by conventional BE of the GH-13 family.

The oligomeric structure of the protein was also determined.

The molecular mass determined by gel filtration chromatography (55.8 kDa) was similar to the subunit molecular mass (66.1 kDa), suggesting that the TK1436ΔH protein exists as a monomer.

DISCUSSION

In this study, we have identified a novel BE of the GH-57 family (GH-57-type BE) through characterization of the TK1436 protein. The GH-57 family was defined in 1996 as a novel α -amylase group (14), structurally distinct from the GH-13 family (18). The characterized reactions of the GH-57

FIG. 4. Reaction products of the TK1436 Δ H protein acting on amylose AS-30. The reaction mixture was incubated at 70°C for the times shown, and the products were analyzed by HPLC. P1, P2, and P3 correspond to peak 1, peak 2, and peak 3, respectively (see the text).

family members (such as α -amylase, 4- α -glucanotransferase, and amylopullulanase) mainly use α -glucans as a substrate, although α -galactosidase specificity has also been reported (43). The present identification of BE specificity in the GH-57 family is very significant, because this finding proves the existence of all four basic activities in the GH-57 family pertaining to synthesis and decomposition of α -glucans (i.e., hydrolysis of α -1,4- and α -1,6-glucosidic linkages and transglycosylation to form α -1,4- and α -1,6-glucosidic linkages). It thus seems that the GH-57 family can be regarded as another “ α -amylase family” with a structural background distinct from that of the GH-13 family.

The results in this paper allowed us to identify GH-57-type BEs in a number of microorganisms (Table 2). Originally, these proteins were annotated as “probable α -amylase” or “glycoside hydrolase.” In spite of the present identification of BE specificity in an archaeal protein, distribution of the GH-57-type BEs in the archaeal domain is limited to the order *Thermococcales*. On the other hand, a large number of orthologs are present in the bacterial domain. In particular, it is intriguing that all of the completely sequenced cyanobacteria contain a GH-57-type BE gene in addition to the conventional

GH-13-type BE gene. It has been reported that a mutant of *Synechocystis* sp. lacking a GH-13-type BE gene still produced α -glucan with an appreciable level of α -1,6 branches, although the source of the branch formation was unclear (44). Now, we assume that GH-57-type BE of the *Synechocystis* sp. is actually functional *in vivo* and that it is responsible for the branched formation.

The GH-57-type BE gene is also distributed in all five sequenced *Mycobacterium* genomes and in both of the *Thermus* genomes available. On the other hand, while most species of *Bacillus* and *Clostridium* contain a GH-13-type BE gene, only a GH-57-type BE gene is present in *Bacillus halodurans* and *Clostridium acetobutylicum*. In these organisms, it may be that genes encoding GH-57-type BE were replaced with genes encoding GH-13-type BE during evolution.

Archaeal species in the order *Thermococcales* seem to synthesize glycogen-like glucans ubiquitously: (i) an α -glucan with a branched structure was found in *Thermococcus hydrothermalis* (11), and a similar glycogen-like glucan was detected in *T. kodakaraensis* KOD1 (36) and *Thermococcus celer* (23); (ii) glycogen synthase (GS) genes are present in all the completely sequenced genomes of *Thermococcales*; and (iii) GS proteins of *Pyrococcus furiosus* and *Pyrococcus abyssi* contain carbohydrate polymerization activities (15, 45). At least in the *Thermococcales*, therefore, the previous absence of a BE ortholog gene in the genome sequence was a “missing link” preventing an understanding of glycogen biosynthesis. BE activity was previously reported in *T. hydrothermalis* cells grown on maltose (11). After partial purification, the authors reported that a 65-kDa protein showed BE activity, although analysis to identify the protein sequence was not performed. As this molecular mass is equivalent to that of the TK1436 Δ H protein (66.1 kDa), it is possible that the BE of *T. hydrothermalis* is a protein devoid of the (HhH)₂ motif, like the PH1386 protein of *Pyrococcus horikoshii*. Alternatively, the (HhH)₂ motif of *T. hydrothermalis* BE might be lost during purification.

Despite the identification of BE, our understanding of the glycogen biosynthesis pathway in the *Thermococcales* is not complete, because glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) has not been identified. In the cases of *P. furiosus* and *P. abyssi*, glycogen can be synthesized through UDP-glucose, because orthologous genes encoding glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) are present in these organisms (PF0770 and PF1356 in *P. furiosus* and PAB0771 in *P. abyssi*), and GS of the *Thermococcales* catalyzes the transfer of glucosyl units from both ADP-glucose and UDP-glucose (11, 15, 45). Nevertheless, as glucose-1-phosphate uridylyltransferase ortholog genes are not ubiquitously present in the *Thermococcales*, we suggest that the *Thermococcales* contain a gene(s), as yet unidentified, encoding glucose-1-phosphate adenylyltransferase.

Presence of the (HhH)₂ motif in the TK1436 protein is interesting, because this motif is found in proteins related to DNA replication or DNA repair (6). In our experiment, deletion of the C-terminal (HhH)₂ motif did not influence the basic enzyme properties (optimum temperature, optimum pH, and specific activity), suggesting that the motif has no substantial role in enzyme reaction. On the other hand, aggregation of wild-type protein was frequently observed when buffers without Triton X-100 were used, while protein devoid of the motif

TABLE 2. Distributions of genes encoding GH-57-type BE and other glycogen biosynthesis enzymes

Organism ^a	Gene encoding:			
	BE (GH-57)	BE (GH-13)	GPA ^b	GS
<i>Archaea: Thermococcales</i>				
<i>Thermococcus kodakaraensis</i> KOD1	TK1436	ND ^c	ND	TK1768
<i>Pyrococcus furiosus</i> DSM 3638	PF1393	ND	ND	PF2044
<i>Pyrococcus horikoshii</i> OT3	PH1386	ND	ND	PH0069
<i>Pyrococcus abyssi</i> GE5	PAB1857	ND	ND	PAB2292
<i>Bacteria: Cyanobacteria</i>				
<i>Gloeobacter violaceus</i> PCC7421	glr4151	glr1306	gll4260	glr0932
<i>Thermosynechococcus elongates</i> BP-1	tl11974	tl10578	tlr1287	tl10763
<i>Synechococcus</i> sp. strain PCC6301	syc2206_d	syc0464_c	syc0921_d	syc1589_d
<i>Prochlorococcus marinus</i> SS120	Pro1135	Pro1078	Pro0842	Pro1052
<i>Synechocystis</i> sp. PCC6803	sl10735	sl10158	slr1176	sl10945, sl11393
<i>Anabaena</i> sp. PCC7120	alr2450	all0713	all4645	alr0031, alr1879
<i>Bacteria: Actinobacteria</i>				
<i>Mycobacterium leprae</i> TN	ML1714	ND	ML1069	ND
<i>Mycobacterium tuberculosis</i> H37Rv	Rv3031	Rv1326c	Rv1213	ND
<i>Mycobacterium tuberculosis</i> CDC1551	MT3115	MT1368	MT1251	ND
<i>Mycobacterium bovis</i> AF2122/97	Mb3057	Mb1361c	Mb1245	ND
<i>Mycobacterium avium</i> K-10	MAP3063	MAP2434	MAP2564c	ND
<i>Nocardia farcinica</i> IFM 10152	nfa42810	nfa10780	nfa47260	ND
<i>Bacteria: Deinococcus-Thermus</i>				
<i>Thermus thermophilus</i> HB8	TTHA1902	ND	TTHA0022	TTHA0018
<i>Thermus thermophilus</i> HB27	TTC1540	ND	TTC1976	TTC1980
(<i>Deinococcus radiodurans</i> R1)	ND	DR1848	DR1689, DR1824	DR0594
<i>Bacteria: Bacillales</i>				
<i>Bacillus halodurans</i> C-125	BH1415	ND	BH1086, BH1087	BH1085
(<i>Bacillus subtilis</i> 168)	ND	BG10907	BG10908, BG10909	BG10910
(<i>Bacillus cereus</i> ATCC 14579)	ND	BC4867	BC4866	BC4864
<i>Bacteria: Clostridia</i>				
<i>Clostridium acetobutylicum</i> ATCC 824	CAC2414	ND	CAC2237, CAC2238	CAC2239
(<i>Clostridium perfringens</i> 13)	ND	CPE0063, CPE1588	CPE0068, CPE0069	CPE0064
(<i>Clostridium tetani</i> E88)	ND	ND	CTC02215	ND

^a Organism name in parentheses indicates the absence of TK1436 ortholog genes.^b GPA, glucose-1-phosphate adenyltransferase.^c ND, not detected.

was stable in buffers without Triton X-100 (unpublished data). From these results, we think that the (HhH)₂ motif of the TK1436 protein constitutes an additional domain through which the TK1436 protein is attached to other substances. Although further experiments are necessary, one possibility is that the motif works as a binding module to α -glucan, just like a plant BE that attaches to small newborn starch granules (31). Alternatively, the motif may be related to the stability of the protein (other than thermostability), as in the case of multiple copies of HhH motifs in *Methanopyrus kandleri* DNA topoisomerase V that are responsible for salt tolerance (4, 30).

In the structural genomics project of *Thermus thermophilus* HB8 (<http://www.thermus.org/>), the crystal structure of a GH-57-type BE ortholog, the TT1467 protein, was determined, and the data are filed in the Protein Data Bank (accession number 1UFA). At present, TT1467 is called TTHA1902. As TK1436 shares 44% amino acid identity with TTHA1902, we performed comparative modeling to predict the 3D structure of the TK1436 protein. Figure 5 shows a model structure around proposed catalytic residues, Glu¹⁸³ (a nucleophile) and Asp³⁵⁴ (an acid/base catalyst). Carboxyl groups from the two catalytic

residues are positioned face to face. The distance between these carboxyl groups (i.e., the average distance separating four pairs of O atoms in the carboxyl groups) is calculated as 6.3 Å. This length is close to that (6.7 Å) of *T. litoralis* 4- α -glucanotransferase, whose 3D structure is the only one reported in the GH-57 family (17). We found five residues (His¹⁰, His¹⁴⁵, Trp²⁷⁰, Trp⁴⁰⁷, and Trp⁴¹⁶) near the catalytic residues. These residues are all conserved in the TK1436 orthologs, along with two catalytic residues. His¹⁰ is a residue included in region A (Table 1). Analysis of substrate-enzyme complexes using *T. litoralis* 4- α -glucanotransferase suggests that the corresponding His residue (His¹³ in *T. litoralis* 4- α -glucanotransferase numbering) is located at the subsite -1 position (17). His¹⁴⁵ is also located near subsite -1 in the predicted structure. Two hydrophobic amino acids, Trp⁴⁰⁷ and Trp⁴¹⁶, are located close to Glu¹⁸³ (a nucleophile) and appear to cover the residue. Therefore, it is predicted that these residues function to prevent acceptor sugar (and water) from approaching from this direction. Unlike the situation with these four residues, Trp²⁷⁰, located in region D, is conserved

FIG. 5. Catalytic-center model of the TK1436 protein. A 3D structural model of TK1436 protein was generated using the SWISS-MODEL Protein Modeling Server. Glu¹⁸³ (a nucleophile) and Asp³⁵⁴ (an acid/base catalyst) are the two catalytic residues conserved in the members of the GH-57 family. Residues (His¹⁰, His¹⁴⁵, Trp²⁷⁰, Trp⁴⁰⁷, and Trp⁴¹⁶) located within 5 Å of either of the carbon atoms in the catalytic carboxyl groups are indicated, while residues next to the catalytic residues are not shown. The yellow dotted line indicates the shortest distance between carbon atoms in the two catalytic carboxyl residues.

only in the TK1436 subfamily, suggesting that the residue has an important function in defining BE specificity.

Overall, the present identification of GH-57-type BEs is noteworthy, since it is the first BE found and characterized outside of the GH-13 family. The discovery of an overlooked BE will contribute significantly to our understanding of a diversified glycogen biosynthesis in living organisms. Moreover, the finding is also important from the viewpoint of industrial application. So far, GH-13-type BEs are reported to be useful for applications such as changing the solubility of starch (21, 38, 40) or producing large cyclic glucans (41, 42). Therefore, it is expected that GH-57-type BEs also have the potential to be applied in the starch-processing industry.

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