The hyperthermophilic archaeon Thermococcus kodakaraensis KOD1 possesses chitinase (Tk-ChiA) and exo-β-N-glucosaminidase (Tk-GlmA) for chitin degradation; the former produces diacetylchitobiose (GlcNAc₂) from chitin, and the latter hydrolyzes chitobiase (GlcN₂) to glucosamine (GlcN). To identify the enzyme that physiologically links these two activities, here we focused on the deacetylase that provides the substrate for Tk-GlmA from GlcNAc₂. The deacetylase could be detected in and partially purified from T. kodakaraensis cells, and the corresponding gene (Tk-dac) was identified on the genome. The deduced amino acid sequence was classified into the LmbE protein family including N-acetylglucosaminylphosphatidylinositol deacetylases and N-acetylglucosaminidase. Recombinant Tk-Dac showed deacetylase activity toward N-acetylchitooligosaccharides (GlcNAc₃₋₄), and the deacetylation site was revealed to be specific at the nonreducing GlcNAc residue. The enzyme also deacetylated GlcNAc monomer. In T. kodakaraensis cells, the transcription of Tk-dac, Tk-glmA, Tk-chiA, and the clustered genes were induced by GlcNAc₂, suggesting the function of this gene cluster in chitin catabolism in vivo. These results have revealed a unique chitin catabolic pathway in T. kodakaraensis, in which GlcNAc₂ produced from chitin is degraded by the concerted action of Tk-Dac and Tk-GlmA. That is, GlcNAc₂ is site-specifically deacetylated to Glcn-GlcNAc by Tk-Dac and then hydrolyzed to GlcN and GlcNAc by Tk-GlmA followed by second deacetylation step of the remaining GlcNAc by Tk-Dac to form GlcN. This is the first elucidation of an archaeal chitin catabolic pathway and defines a novel mechanism for dimer processing using a combination of deacetylation and cleavage, distinct from any previously known pathway.

Chitin is the linear homopolymer of β-1,4-linked N-acetylglucosamine (GlcNAc), and its biological production is the most abundant after cellulose. Degradation of chitin in eucaryotes and bacteria has been studied very well, and chitin catabolic pathways clarified from these studies are summarized in Fig. 1A (thin arrows). Chitin is degraded into diacetylchitobiose (GlcNAc₂) by the combination of endo- and exo-type chitinases (reactions 1 and 2) followed by dimer processing with β-N-acetylglucosaminidase (GlcNAcase; reaction 3), GlcNAc₂ phosphorylase (reaction 4), or GlcNAc₂ phosphotransferase system (reaction 5) and 6-phospho-β-glucosaminidase (reaction 6) (1–4). In these pathways removal of the N-acetyl group derived from the starting chitin occurs after the degradation to monomers. This step is catalyzed by GlcNAc-6-phosphate (GlcNAc6P) deacetylase, which deacylates the GlcNAc6P produced by cleavage of GlcNAc6P-GlcNAc (reaction 6) or by phosphorylation of GlcNAc. An alternative pathway for chitin degradation is proposed to be initiated by deacetylation of chitin by chitin deacetylase (reaction 7). The resulting deacetylated chitin, chitosan, is then degraded to glucosamine (GlcN) by chitosanase (endo-type enzyme; reaction 8) in cooperation with exo-β-N-glucosaminidase (GlcNase; reaction 9) (1).

In contrast to eucaryotes and bacteria, there is very little information concerning chitinolysis in archaea. We previously reported the first characterization of a thermostable chitinase from a hyperthermophilic archaeon, Thermococcus kodakaraensis KOD1 (5, 6). The chitinase (Tk-ChiA) has a unique domain structure that is composed of dual catalytic domains and triple chitin binding domains. The dual catalytic domains can individually cleave chitin chains with different profiles, namely, the N- and C-terminal catalytic domains exhibit exo- and endo-type cleavage specificities, respectively. Other archaeal chitinases from the hyperthermophiles Thermococcus chitonophasus (7) and Pyrococcus furiosus (8) have also been reported. These chitinases from hyperthermophilic archaea, including Tk-ChiA, produce GlcNAc₂ as an end product from chitin. Recently, we have identified another chitinolytic enzyme, GlcNase, from T. kodakaraensis in the course of searching for enzymes involved in GlcNAc₂ catabolism (9). This GlcNase (Tk-GlmA) hydrolyzed chitobiase (GlcN₂) to GlcN and was induced by GlcNAc₂, the end product from chitin by Tk-ChiA. These facts have suggested the presence of a GlcNAc₂-specific deacetylase activity in T. kodakaraensis in order to supply the substrate for Tk-GlmA from GlcNAc₂ (Fig. 1A, thick arrows).
In this study we performed purification and characterization of a GlcNAc deacetylase (Tk-Dac) from T. kodakaraensis and have clarified that one gene with previously unknown function encoded this protein. Tk-Dac deacetylated the nonreducing terminal unit of N-acetylglucosaminocarbohydrates as well as GlcNAc monomers, constituting a novel archaeal chitin catabolic pathway in this organism together with Tk-ChiA and Tk-GlmA (Fig. 1B).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media—**T. kodakaraensis KOD1 was grown anaerobically for 48 h at 65 °C in 1-liter screw-cap bottles with 800 ml of MA medium (4.8 and 26.4 g of Marine Art SP agents A and B, respectively (Senju Seiyaku, Osaka, Japan), 5 g of yeast extract, and 5 g of Tryptone in 1 liter of deionized water) supplemented with pyruvic acid sodium salt (5 g/liter) and chitin (1 g/liter). Escherichia coli TG-1 and BL21-CodonPlus(DE3)-RII were used as hosts for the expression plasmid derived from pET-15b (Novagen, Madison, WI) and were cultivated in LB medium at 37 °C.

**Partial Purification of GlcNAc Deacetylase from T. kodakaraensis KOD1—**The culture broth of T. kodakaraensis containing chitin (11.2 liters) was filtered through Toyo filter paper No. 101 (Toyobo, Tokyo, Japan) to obtain chitin-associated cells. The cells on the chitin particles were suspended in buffer A (50 mM Tris-HCl (pH 8.0), 1 mM EDTA) and then disrupted by sonication. The supernatant obtained by centrifugation (6000 × g for 15 min at 4 °C) was subjected to ammonium sulfate fractionation, and the 50–70% ammonium sulfate-precipitated fraction was dissolved in 45 ml of buffer A. The protein solution was applied to an anion-exchange Mono Q HR 5/5 column equilibrated with buffer B. The peak fractions were dialyzed against buffer C (20 mM Tris-HCl (pH 7.5)) and applied to an anion-exchange Mono Q HR 5/5 column equilibrated with buffer B. The peak fractions were dialyzed against buffer D containing chitin (11.2 g) and eluted with a linear gradient of 0.2–0.5 M NaCl. Fractions containing GlcNAc deacetylase activity were collected and concentrated using Ultrafree-4 centrifugal filter unit Biomax-30 (Millipore, Bedford, MA).

The sample was then applied to a gel-filtration Superdex-200 HR 10/30 column (Amersham Biosciences) equilibrated with buffer B (50 mM Tris-HCl (pH 8.0), 150 mM NaCl). The collected active fractions were dialyzed against buffer C (50 mM Tris-HCl (pH 7.5)) and applied to an anion-exchange Mono Q HR 5/5 column (Amersham Biosciences). The proteins were eluted with a linear gradient of 0.2–0.5 M NaCl. Ammonium sulfate was added to the active fractions after the Mono Q column at a final concentration of 1.5 M, and the sample was applied onto hydrophobic Resource ISO (1 ml) (Amersham Biosciences) that had been equilibrated with 1.5 M ammonium sulfate in 50 mM Tris-HCl buffer (pH 8.0). The proteins were eluted with a linear gradient of 1.5–0.5 M ammonium sulfate. The active fractions were combined and used for activity staining after polyacrylamide gel electrophoresis. Protein concentration was determined with the Bio-Rad protein assay system with bovine serum albumin as a standard.

**Activity Staining—**GlcNAc deacetylase activity was detected in the gel after non-denaturing SDS-PAGE by using the fluorogenic substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (GlcNAc-4MU, Sigma) with recombinant exo-β-N-glucosaminidase from T. kodakaraensis (Tk-GlmA) (9) as a coupling enzyme. A protein sample mixed with 2× sample buffer was applied to 12.5% SDS-polyacrylamide gel without prior boiling. After the electrophoresis, the gel was rinsed three times with 100 mM Tris-HCl (pH 8.0) for 20 min followed by soaking in the reaction buffer (100 μl GlcNAc-4MU, 100 mM Tris-HCl (pH 8.0)) at room temperature for 30 min. The gel removed from the reaction buffer was overlaid with a Tk-GlMA-containing gel (10 ng recombinant Tk-GlMA, 12.5% acrylamide, 0.33% N,N'-methylbisacrylamide, 100 mM Tris-HCl (pH 8.0), 0.25% ammonium persulfate, 0.05% TEMED), and the fluorescent band was visualized on a transilluminator (312 nm) after incubation of the gel bilayer at 70 °C for 5–15 min. The N-terminal amino acid sequence of the active protein was determined by a protein sequencer (Model 491 LC Applied Biosystems, Foster City, CA).

DNA Manipulations and Sequencing—DNA manipulations were carried out by standard methods, as described by Sambrook and Russell (10). Restriction enzymes and other modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan). Small scale preparation of plasmid DNA from E. coli cells was performed with the Qiagen plasmid Mini kit (Qiagen, Hilden, Germany). DNA sequencing was performed with BigDye Terminator Cycle Sequencing Ready Reaction kit Version 3.0 and Model 3100 capillary DNA sequencer (Applied Biosystems).

**Construction of the Expression Plasmid—**The expression plasmid for Tk-dac was constructed by PCR as described below. Two oligonucleotides (sense, 5'-TGGCGATCCGGTGTTTGAGGAGTTCAAC-3'; antisense, 5'-GGCCAGGATCCGAGGAGGTACAG-3') (underlined sequences indicate an NcoI site in the sense primer and a BamHI site in the antisense primer, respectively) and T. kodakaraensis genomic DNA were used as primers and template for DNA amplification, respectively. The amplified DNA was digested with NcoI and BamHI and then ligated with the corresponding sites in the plasmid pET-15b. The absence of unintended mutations in the insert was confirmed by DNA sequencing. The resulting plasmid was designated as pET-dac.

**Purification of Recombinant Tk-Dac—**E. coli BL21-CodonPlus(DE3)-RII cells harboring pET-dac were induced for overexpression with 0.05 mM isopropyl-β-D-thiogalactopyranoside at the mid-exponential growth phase and incubated for a further 3 h at 37 °C. The cells were harvested by centrifugation (5000 × g for 15 min at 4 °C), resuspended in buffer D (50 mM Tris-HCl (pH 7.5), 1 mM EDTA), and then disrupted by sonication. The supernatant after centrifugation (14,000 × g for 30 min) was incubated at 80 °C for 20 min and centrifuged (14,000 × g for 15 min) to obtain a heat-stable protein solution. The solution was applied to ammonium sulfate precipitation at 70% saturation, and the resulting precipitate was dissolved in 1.3 M ammonium sulfate in buffer E (50 mM Tris-HCl (pH 8.0), 1 mM EDTA). Insoluble proteins were removed by centrifugation (28,000 × g for 15 min at 4 °C), and the supernatant was applied to a hydrophobic Resource ISO column (6 ml) (Amersham Biosciences) equilibrated with 0.9 M ammonium sulfate in buffer E. The proteins were eluted with a linear gradient of 0.9–0.45 M ammonium sulfate, and the peak fractions eluting at 0.85 M ammonium sulfate were concentrated using Ultrafree-4 centrifugal filter unit Biomax-10 (Millipore). This was applied to a gel-filtration Superdex-200 HR 10/30 column equilibrated with buffer B. The peak fractions were dialyzed against buffer C and applied to an anion-exchange Mono Q HR 5/5 column. The proteins were eluted with a linear gradient of 0.2–0.5 M...
NaCl, and the peak fractions were dialyzed with 10 mM Tris- HCl (pH 7.5) and stored at 4 °C.

**Enzyme Assays**—Deacetylase activity during purification of the native enzyme was assayed with a fluorogenic substrate, GlcNAc-4MU, under the presence of excess recombinant Tk-GlmA for coupled hydrolysis. The reaction was performed at 70 °C for 60 min in 500 μl of 10 μM GlcNAc-4MU, 10 nM Tk-GlmA, and 50 mM Tris-HCl (pH 8.0). The reaction was terminated by cooling the sample in an ice-cold bath. To determine the optimal pH for recombinant Tk-Dac, the reaction was performed for 10 min at 70 °C in 250 μl of 10 μM GlcNAc-4MU, 4 nM recombinant Tk-Dac, and 50 mM appropriate buffer (citrate-NaOH, HEPES-NaOH, or CHES-NaOH). After cooling the sample to stop the reaction, the protein was removed from the reaction mixture with a centrifugal filter (Microcon YM-10). Removal of the Tk-Dac protein was confirmed by the absence of detectable deacetyl activity in the filtrate. 100 μl of the filtrate was then added into 400 μl of 12.5 nM Tk-GlmA and 50 mM MES-NaOH (pH 6.0). This mixture was incubated at 70 °C for 10 min. This reaction time (10 min) was confirmed to be sufficient to complete the second reaction under the presence of excess Tk-GlmA. In the case of determining optimal temperature, the first reaction was performed in 50 mM HEPES-NaOH (pH 8.5) at various temperatures (37–100 °C). To quantify the liberated 4-methylumbelliferyl, the cooled sample (500 μl) was mixed with 500 μl of 100 mM glycine-NaOH (pH 11), and the fluorescence (350 nm, excitation; 440 nm, emission) was measured with a spectrofluorometer (model F-2000; Hitachi, Tokyo, Japan).

Kinetic properties of recombinant Tk-Dac toward N-acetylchitooligosaccharides were determined as below. The reactions were performed with various concentrations of GlcNAc, 1–5 μM; GlcNAc, 5–160 μM; GlcNAc, 2.5–80 μM; GlcNAc, 1.25–40 μM; and 50 mM MES-NaOH (pH 8.5) at 75 °C for 5 min. The reactions were stopped by adding 10 μl of 0.3 M HCl at 4 °C, and then Tk-Dac was removed with Microcon YM-10. The amount of acetic acid in the filtrate was enzymatically determined by using an acetic acid determination kit (Roche Diagnostics). Bulk GlcNAc and GlcNAc were kindly donated from Yaizu Suisankagaku Industry (Shizuoka, Japan). Acetic acid slightly contaminated in these substrates had been removed before use by an anion-exchange spin column (NAP-10 column, Mini H; Sartorius, Goettingen, Germany).

**Analyses of Reaction Products**—The analyses of reaction products from GlcNAc, 1–5; N-acetylglactosamine, N-acetylmannosamine, N-acetylmuramic acid, and GlcNAc6P were performed with silica gel thin-layer chromatography (TLC) as described previously (5) except that we used an LHP-KF HPTLC plate (Whatman, Kent, UK) in this study. For detection of the products, aniline diphénylamide reagent and ninhydrin reagent were used.

**Western Blot Analysis**—T. kodakaraensis was cultivated in 10 ml of MA medium supplemented with 20 μl of polysulfide solution (20% elemental sulfur in 3 mM Na2S) and various kinds of saccharides. The preparation of colloidal chitin has been described previously (5). The cell pellets were disrupted by sonication in buffer C containing protein inhibitor mix (Complete mini; Roche Diagnostics), and then centrifuged (15,000 × g for 30 min) to obtain soluble fractions. Each fraction was subjected to SDS-PAGE and Western blot analysis using specific antiserum (rabbit) against the recombinant Tk-Dac. A protein A-peroxidase conjugate was used to visualize the specific protein. The reaction was performed at 70 °C for 4 min in 50 mM MES-NaOH, 8.1 mM Na2HPO4, 2.68 mM KCl, 1.47 mM KH2PO4, and the addition of 100 μl of the cell extract (2 mg of protein/ml) of T. kodakaraensis grown in the medium containing 0.1% GlcNAc2. After incubation at 4 °C for 1 h, the supernatant was subjected to determine the GlcNAcase, GlcNase, and deacetylase activities.

**RESULTS**

**Identification of the Protein Exhibiting GlcNAc2 Deacetyl Activity and the Corresponding Gene in T. kodakaraensis**

**KO1**—As described above we have previously demonstrated that T. kodakaraensis possesses a chitinase (Tk-Chia) for degradation of chitin polymer to GlcNAc along with a novel exo-β-d-glucosaminidase (Tk-GlmA) to cleave the deacetylated product of GlcNAc2 (5, 6, 9). These results strongly suggested the existence of an enzyme responsible for the deacetylation of GlcNAc2 in this organism. Therefore, the deacetylase activity in T. kodakaraensis was examined by using a fluorogenic GlcNAc-4MU and recombinant Tk-GlmA as a substrate and a coupling enzyme, respectively. It has been already clarified that Tk-GlmA cannot cleave this substrate due to the N-acetyl group on the sugar moiety nor can it deacetylate this substrate (9). The cell extract of T. kodakaraensis showed only weak cleavage activity (apparent GlcNAcase activity) in the absence of the recombinant Tk-GlmA (4.16 pmol/min/mg). In contrast, the addition of the coupled enzyme enhanced the cleavage activity up to 5.5-fold (23.1 pmol/min/mg), indicating a significant deacetylase activity in the extract. We then performed purification of the protein exhibiting this deacetylase activity from T. kodakaraensis cells. The cells cultivated in chitin-containing medium were disrupted by sonication, and the active protein was partially purified by ammonium sulfate fractionation and column chromatography, as described under “Experimental Procedures.” Although many protein bands were still observed in non-denaturing SDS-PAGE after the final chromatography (Fig. 2A, lane 2), a protein with GlcNAc2 deacetylase activity in the gel could be identified by activity staining using GlcNAc-4MU and recombinant Tk-GlmA (Fig. 2A, lane 1, black arrowhead). In the negative control experiment without the coupling enzyme, the deacetylase-active band was not detected (data not shown).

The N-terminal amino acid sequence of the identified protein was determined to be VFEEFNPDFEA. Using the preliminary complete genome sequence of T. kodakaraensis KO1, we searched for this amino acid sequence and identified one open reading frame that encoded a protein with the N-terminal sequence of MYFEFNPDFEA (identical amino acids are underlined). Interestingly, this gene was located adjacent upstream of the Tk-chia gene in the opposite orientation and also relatively near (−9 kilobase pairs upstream) Tk-glmA (Fig. 3). This gene was designated as Tk-dac.

**Primary Structure of Tk-dac**—The Tk-dac gene consisted of 804 bp, encoding a protein of 268 amino acids with a predicted molecular mass of 30,300 Da. There was neither an N-terminal signal sequence nor membrane helices in the deduced amino acid sequence, which was consistent with the fact that Tk-Dac was detected and partially purified from the cytosolic fraction of T. kodakaraensis cells as an intracellular enzyme. Clear-cut homologs against the translated product were only seen in the closely related Pyrococcus spp., Pyrococcus abyssi.
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T. kodakaraensis in Fig. 2A. The molecular mass of the recombinant Tk-Dac was estimated to be about 30 kDa by denaturing SDS-PAGE and 160 kDa by gel filtration chromatography (figure not shown), and the latter was comparable with that of the active protein from T. kodakaraensis. The results indicated that Tk-Dac was likely to be a homohexameric enzyme.

Enzymatic Properties of Tk-Dac—The optimal pH and temperature of Tk-Dac for GlcNAc-4MU were 8.5 and 75 °C, respectively. Activity levels at 37 °C and 100 °C were ~20% of that observed at the optimal temperature. To examine the mode of action of this enzyme, various chain lengths of N-acetylmuramoyl carbohydrates (GlcNAc₂₋₅) were used as substrates, and the reaction products were analyzed by TLC with two detection procedures. The results are shown in Fig. 4. Tk-Dac could efficiently deacetylate GlcNAc to GlcN. When GlcNAc₂₋₅ were used as substrates, detection of the products with ninhydrin reagent (for amino sugars) (Fig. 4A) clearly indicated formation of deacetylated products from all the substrates examined. However, the mobilities of each reaction product did not coincide with those of completely deacetylated molecules, GlcN₂₋₅, suggesting partial deacetylation at specific position(s). We applied Tk-GlmA, which specifically cleaves the first β-1,4-glycosidic bond from the nonreducing end of GlcN oligomers (9), to clarify the site specificity of Tk-Dac. In this experiment GlcNAc₂₋₅ were completely converted with Tk-Dac in the initial reaction, and then Tk-GlmA was added to the reaction mixture after the removal of Tk-Dac. These samples were subjected to TLC analysis, and the results are shown in Fig. 5. With the second reaction with Tk-GlmA, the deacetylated products after the first Tk-Dac reaction (Fig. 5B) were found to be degraded to GlcN and N-acetylmuramoyl carbohydrates (GlcNac₈₋₅), one unit shorter than the starting substrates (GlcNac₉₋₅) (Fig. 5C). These results clarified that Tk-Dac specifically deacylated the nonreducing end unit of GlcNAc₂₋₅. Considering the acceptance of GlcNAc monomer as a substrate for Tk-Dac, GlcNAc oligomers can be completely converted to GlcN monomers by the reciprocal actions of Tk-Dac and Tk-GlmA. It should be noted that the deacetylation activity of Tk-Dac toward other N-acetylmuramoyl saccharides (N-acetylgalactosamine, N-acetylmuramic acid, and GlcNAc6P) was not observed except for a faint activity toward N-acetylmannosamine (data not shown), suggesting a high specificity of this enzyme toward the GlcNAc moiety for deacetylation.

We then carried out kinetic analysis toward GlcNAc₁₋₃ by determining the amount of acetic acid released by Tk-Dac. The enzyme followed Michaelis-Menten kinetics and exhibited the highest V_max value toward GlcNAc and the lowest K_max value toward GlcNAc₁ among these substrates (Table I). The resulting k_cat/K_m ratios indicated that GlcNAc and GlcNAc₁ were favorable substrates for Tk-Dac, whereas the value toward GlcNAc₁ was 1 order lower.

Expression profiles of Tk-Dac in T. kodakaraensis—The expression of Tk-Dac in T. kodakaraensis cells grown in media containing various sugars with elemental sulfur was examined by Western blot analysis (Fig. 6). The expression of Tk-Dac was not observed under basal culture condition (lane 1), whereas it was obviously induced by the addition of GlcNAc₂, an end product from chitin by Tk-ChiA (lane 3). In contrast, GlcNAc, a substrate comparable with GlcNAc₂ for Tk-Dac, could not act as an inducer (lane 4). The addition of other non-N-acetylated saccharides (GlcN, lactose, cellobiose, maltose, and GlcN) also resulted in no induction except for a faint signal with GlcN₂ (data not shown). The specific induction by GlcNAc suggested the participation of Tk-Dac in the chitin degradation pathway. We have previously reported that Tk-GlmA was weakly induced with colloidal chitin after prolonged cultivation for 72 h,
probably due to the accumulation of GlcNAc₂ derived from chitin (9). A similar induction profile was also observed for Tk-Dac, although the protein expression was only just enough to detect even after the prolonged 72-h cultivation (lane 7).

As shown in Northern blot analysis for Tk-dac (Fig. 7A, lane 7), the transcription was specifically induced by GlcNAc₂, as expected from the results of Western blot analysis. However, we failed to detect the signal under the supplement of colloidal chitin even after prolonged cultivation (51 h), probably due to the weak transcription and significant degradation of RNA at the stationary phase (Fig. 7A, lanes 4 and 5). We, therefore, performed the more sensitive RT-PCR analysis for Tk-dac as well as for other genes clustered with Tk-dac (Tk-GlmA, Tk-Gly, and Tk-ChiA; see Fig. 3) to investigate the transcriptional regulation of these clustered genes (Fig. 7B). We confirmed that no signal was detected in control experiments without the RT reaction in all combinations of RNA samples and primers for PCR and that the amount of PCR products did not saturate during the PCR reactions (data not shown). As a result, the transcription of all examined genes was highly induced by GlcNAc₂ (Fig. 7B, lane 12). Moreover, although the intensities were much weaker, specific induction by colloidal chitin could be detected for the genes both at growth and stationary phases (Fig. 7B, lanes 13 and 15). Tk-dac and other clustered genes were transcriptionally induced under chitin degradation conditions in the same manner, where the degradation intermediate, GlcNAc₂, was supposed to potentially act as a direct inducer. These results strongly suggest the possible function of this gene cluster in chitin degradation.

Absence of GlcNAcase in T. kodakaraensis—As described above, the cell extract of T. kodakaraensis exhibited a weak GlcNAcase activity when GlcNAc-4MU was applied as a substrate. This apparent GlcNAcase activity could be accounted for by the combination of Tk-Dac and Tk-GlmA. However, a possibility of the existence of another protein(s) responsible for the GlcNAcase activity still remained. From this viewpoint, we examined the individual enzyme activities in the cell extract
after immunoprecipitation using specific antiserum against recombinant Tk-Dac or Tk-GlmA (Table II). When the cell extract from GlcNAc₂−induced Tk. kodakaraensis was treated with non-specific antiserum, GlcNAsc activity was detected along with GlcNase and deacetylase activities. In contrast, treatments with antiserum against Tk-GlmA or Tk-Dac, both resulted in abolishment of the GlcNAsc activity accompanied by removal of the corresponding activity. Considering these facts together with the catalytic properties of the particular enzymes, the GlcNAsc activity in the extract can be concluded as a result of the concerted action of Tk-GlmA and Tk-Dac, and no other protein was responsible for this apparent activity.

**DISCUSSION**

This study was initiated to identify the protein responsible for the missing enzyme activity between the previously reported chitinase (Tk-ChiA) and exo-β-1,4-glucosaminidase (Tk-GlmA) involved in the chitin catabolic pathway of the hyperthermophilic archaeon *Tk. kodakaraensis* KOD1. As previously estimated, we successfully identified a novel GlcNAsc deacetylase and its gene (Tk-dac). It is notable that the Tk-dac gene was clustered together within a 13-kilobase pair region with Tk-chiA, Tk-glmA, and genes for putative ABC transporter components on the genome of *Tk. kodakaraensis* (Fig. 3).

The action of Tk-Dac was site-specific; that is, the deacetylation of *N*-acetylchitooligosaccharides (GlcNAsc₂₋₃) by this enzyme occurred at the nonreducing end (Figs. 4 and 5). Although our investigation for the chain length specificity was limited, GlcNAsc₂ was a better substrate than GlcNAsc₃ for Tk-Dac, and additionally, this enzyme could also deacetylate the monosaccharide, GlcNAsc, with a similar kcat/Km ratio to that for GlcNAsc₂ (Fig. 4 and Table I). These catalytic properties and our previous findings indicate a unique chitin catabolism in *Tk. kodakaraensis* (Fig. 1B). Tk-GlmA is produced from chitin by extracellular Tk-ChiA and is likely to be imported into the cells by the ABC transporter encoded in the cluster. Within the cells the GlcNAsc₂ is specifically deacetylated at the nonreducing GlcNAsc residue by Tk-Dac. The partially deacetylated disaccharide, GlcN-GlcNAsc, is then hydrolyzed to GlcN and GlcNAsc monomers by Tk-GlmA. Finally, GlcNAsc is deacetylated by the second action of Tk-Dac, resulting in complete conversion of chitin to GlcN monomers. The common transcriptional regulation observed for the clustered genes under chitin degradation conditions and the localization of each enzyme well support the proposed in vivo function of the concerted action of Tk-GlmA and Tk-Dac.
this gene cluster. Besides the combination of Tk-GlmA and Tk-Dac, no other enzyme possessing GlcNacase activity was present in T. kodakaraensis, as demonstrated by the immunoprecipitation experiment. Until now, it has been known that chitin is degraded to dimer units by chitinases followed by cleavage to monomers before deacetylation. Alternatively, chitin can be degraded by chitosanase and GlcNase after the initial deacetylation of chitin (Fig. 1A, thin arrows) (1–4). The cleavage of dimer units concerned with deacetylation in T. kodakaraensis is quite distinct from the known pathways in other organisms.

With respect to the primary structure, Tk-Dac belonged to the LmbE-like protein family (Pfam02585, COG2120) including N-acetylglucosaminylphosphatidylinositol de-N-deacetylases and 1-N-myo-inositol-2-acetamido-2-deoxy-D-glucopyranoside deacetylase. Because these enzymes shared a common feature to deacetylate the GlcNac moiety, the other bacterial and archaeal members with unknown function can also be supposed to show deacetylase activity toward various N-acetylglucosaminyll compounds. The recently determined crystal structure of TT1542 from T. thermophilus will contribute to the progress of functional analyses of the proteins in this family. Although many members of this family showed only low similarities to Tk-Dac, the closely related hyperthermophiles Pyrococcus spp. harbored highly homologous proteins. Particularly, P. furiosus possesses diacetylchitocebiase and GlmA orthologs together with two chitinases (related to the N- and C-terminal halves of Tk-ChiA), suggesting the existence of the same archaeal chitinolytic pathway. A previously reported GlcNacase activity in the cytoplasmic fraction of P. furiosus (8) may be derived from a combination of the GlmA and diacetylchitocebiase orthologs, as seen in T. kodakaraensis.

So far chitin deacetylase from fungi and insects, chitoeligosaccharide (NodB) from Rizobium, and bacterial GlcNAc6P deacetylase have been known as catalytically similar deacetylases, all involved in the deacetylation of GlcNac moieties found in chitin and the related saccharides. Chitin deacetylase is capable of randomly removing N-acetyl groups in chitin chains (18). NodB, involved in nodulation signal synthesis, deacetylates the non-reducing GlcNac residue of N-acetylchitoeligosaccharides like Tk-Dac (19); however, it cannot act on the GlcNac monomer. Tk-Dac deacetylated GlcNac monomer as well as oligomers but had no ability to accept GlcNAc6P monomer as a substrate. Moreover, the primary structure of Tk-Dac was not related to those of the known deacetylases above; chitin deacetylase and NodB belong to the polysaccharide deacetylase family (Pfam01522, COG0726), and GlcNAc6P deacetylase belongs to the amidohydrolase family (Pfam01979, COG1820). Apparently, Tk-Dac is a novel enzyme with catalytic properties and primary structure distinguishable from those of the known deacetylases.

In conclusion, we have elucidated that chitin catabolism in T. kodakaraensis is constituted by a unique chitinase possessing dual catalytic domains with different cleavage specificities (5, 6) together with new types of GlcNAc deacetylase (this study) and exo-β-D-glucosaminidase (9). The orthologs for the latter two enzymes had both been annotated as hypothetical proteins with unknown function in previous whole genome analyses of other organisms. Our studies demonstrate that they act in concert in the degradation of the dimer unit generated from chitin and are the first to clarify a novel chitin catabolic pathway in archaea.

### Table II

| Serum used for immunoprecipitation | GlcNacase$^a$ | GlcNase$^b$ | Deacetylase$^c$ |
|------------------------------------|-------------|------------|---------------|
| Serum before immunization          | 0.226 (100%) | 0.218 (100%) | 0.0167 (100%) |
| Anti-Tk-GlmA serum                  | <0.001 (<1%) | 0.007 (2%)   | 0.0170 (102%) |
| Anti-Tk-Dac serum                   | <0.001 (<1%) | 0.411 (113%) | <0.0001 (<1%) |

$^a$ Determined by fluorometric assay with GlcNAc-4MU as a substrate.

$^b$ Determined by method of Tatsuoka et al. (9) with GlcN as a substrate.

$^c$ Determined by enzymatic assay for released acetic acid with GlcNAc as a substrate.

REFERENCES

1. Gooday, G. W. (1994) in Biochemistry of Microbial Degradation (Raitledge, C., ed) pp. 279–312, Kluwer Academic Publishers, Dordrecht, Netherlands

2. Park, J. K., Keyhani, N. O., and Roseman, S. (2000) J. Biol. Chem. 275, 33077–33083

3. Keyhani, N. O., and Roseman, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14367–14371

4. Keyhani, N. O., Wang, L. X., Lee, Y. C., and Roseman, S. (2000) J. Biol. Chem. 275, 33084–33090

5. Tanaka, T., Fujiwara, S., Nishikori, S., Fukui, T., Takagi, M., and Imanaka, T. (1999) Appl. Environ. Microbiol. 65, 5338–5344

6. Tanaka, T., Fukui, T., and Imanaka, T. (2001) J. Biol. Chem. 276, 35629–35635

7. Androutsopoulos, E., and Vorgias, C. E. (2003) Extremophiles 7, 43–53

8. Gao, J., Bauer, M. W., Shuckoll, K. R., Pryz, M. A., and Kelly, R. M. (2003) Appl. Environ. Microbiol. 69, 3119–3128

9. Tanaka, T., Fukui, T., Atomi, H., and Imanaka, T. (2003) J. Bacteriol. 185, 5175–5181

10. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

11. Bateman, A., Birney, E., Cerruti, L., Durbin, R., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M., and Sonnhammer, E. L. L. (2002) Nucleic Acids Res. 30, 276–280

12. Tatsuoka, N. L., Natale, D. A., Garkavtsev, I. V., Tatusova, T. A., Shankavaram, U. T., Rao, B. S., Kryukin, B., Galperin, M. Y., Fedorova, N. D., and Koonin, E. V. (2001) Nucleic Acids Res. 29, 22–28

13. Nakamura, N., Itooe, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V. L., and Kinosita, T. (1997) J. Biol. Chem. 272, 15834–15840

14. Watanabe, R., Ohishi, K., Maeda, Y., Nakamura, N., and Kinosita, T. (1999) Biochem. J. 339, 185–192

15. Chang, T., Milne, R. G., Guth, M. L. S., Smith, T. K., and Ferguson, M. A. J. (2002) J. Biol. Chem. 277, 50176–50182

16. Newton, G. L., Av-Gay, Y., and FAhey, R. C. (2000) J. Biol. Chem. 275, 6958–6963

17. Handa, N., Terada, T., Kanemori, Y., Hamana, H., Tame, J. R. H., Park, S. Y., Kinoshita, O., Mita, N., Nakamura, H., Kuramitsu, S., Shirouzu, M., and Yokoyama, S. (2003) Protein Sci. 12, 1621–1632

18. Tsigis, I., Martinou, A., Kafetzopoulos, D., and Bouriotis, V. (2000) Trends Biotechnol. 18, 305–312

19. John, M., Rührig, H., Schmidt, J., Wienecke, U., and Schell, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 80, 625–629

20. Ezaki, S., Miyao, K., Nishi, K., Tanaka, T., Fujiwara, S., Takagi, M., Atomi, H., and Imanaka, T. (1999) J. Bacteriol. 181, 130–135
Concerted Action of Diacetylchitobiose Deacetylase and Exo-β-D-glucosaminidase in a Novel Chitinolytic Pathway in the Hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1

Takeshi Tanaka, Toshiaki Fukui, Shinsuke Fujiwara, Haruyuki Atomi and Tadayuki Imanaka

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