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Biochemical Characterization of Deblocking Aminopeptidase from Hyperthermophilic Archaeon *Thermococcus onnurineus* NA1

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A genomic analysis of the hyperthermophilic archaeon *Thermoccoccus onnurineus* NA1 (TNA1) revealed the presence of a deblocking aminopeptidase (DAP) gene with high similarity to the genes of DAPs from *Pyrococcus furiosus* (86%) and *Pyrococcus horikoshii* (83% identity). The optimum aminopeptidase activity of the recombinant enzyme was observed at pH 7.5 and in the range of 90°C to 100°C. The specific aminopeptidase and deblocking activities of the enzyme toward Leu-*p*NA and Ac-Leu-*p*NA were 18- and 3-fold higher than those of a *P. horikoshii* DAP (DAP2), respectively. The enzyme activity was significantly increased by Co^{2+} ions. The presence of Co^{2+} ions induced the activation of the enzyme with heating and changed the large oligomer to a dimer. The enzyme activated by Co^{2+} ions appeared to eventually be inactivated by autodegradation, which was confirmed by mass spectrometry.

[**Key words:** *Thermococcus*, deblocking aminopeptidase, hyperthermophile, archaea, autodegradation]

Deblocking aminopeptidases (DAPs; EC 3.4.11.–) act at amino terminals of polypeptide chains and release amino acids from unmodified peptides or peptides modified with various amino-terminal acyl-type blocking groups. The enzymes belong to the M42 peptidase family according to the classification of peptidases in evolutionary clans and families of sequence similarities according to the MEROPS database (1). The M42 peptidase family includes various aminopeptidases from gram-positive bacteria such as the glutamyl aminopeptidase from *Lactococcus lactis* (2) and the thermostable aminopeptidase I from *Bacillus stearothermophilus* (3), and from archaea such as TET from *Haloarcula marismortui* (4) and DAPs from *Pyrococcus furiosus* (5) and *Pyrococcus horikoshii* (6, 7).

All of the characterized M42 peptidases have been reported to show multimeric structures (8), and *H. marismortui* TET and *P. horikoshii* DAP1 and DAP2 display tetrahedral dodecameric architecture (9–11). Self-compartmentalizing quaternary structures with active sites situated in the inner cavity could deveol a central chamber with narrow entry channels, allowing access only to small peptides or unfolded proteins as substrates, as displayed by X-ray crystallography and electron microscopy.

Four archaeal DAPs from *P. furiosus* and *P. horikoshii* have been purified and characterized so far. *P. furiosus* DAP has been commercially used for peptide sequence analysis of the N-terminus acyl peptide, by hydrolyzing not only an N-terminal acyl-type blocking group, but also amino acids successively from N-termini, except the X-Pro bond (5, 12). *P. horikoshii* DAPs have been studied by several research groups, for example, DAP1 (ORF PH0519) (6), DAP2 (ORF PH1527), which is also named FrvX (7, 9, 11), and DAP3 (ORF PH1821) (7). For these DAPs, the substrate specificities and activators varied although the peptidase domains contained conserved binding sites for two catalytic metal ions in the active site, bound by His, Asp, Glu, Asp, and His, and catalytic residues, Asp and Glu (7).

The physiological roles of the DAPs in *Pyrococcus* sp. cells are unknown, but it has been suggested that the self-compartmentalizing quaternary structures of DAPs could contribute to maintaining protein quality together with the ATP-dependent protease system. Furthermore, the roles of these enzymes appear to be related to protein maturation and the metabolism of peptides from proteasomes because DAPs can act only on moderately long polypeptides and have broad substrate specificity (7, 13). The presence of three open reading frames (ORFs) homologous to the genes encoding *P. horikoshii* DAPs in the genome databases of *P. furiosus* and *P. abyssi* suggests that DAPs might work in a complementary manner, requiring different substrate specificities (7, 13), implying that the enzyme activity may play a critical role in maintaining homeostasis. However, the regulation mechanism of the enzyme is not understood yet.

Recently, we isolated the hyperthermophilic archaeon *Thermoccoccus onnurineus* NA1 (14), and the entire genome sequence was determined (Lee *et al.*, unpublished) to search for valuable extremely thermostable enzymes. The analysis of the genome information of *T. onnurineus* NA1 revealed a deblocking aminopeptidase gene homologous to the *P. furiosus* DAP gene. In this study, we describe the cloning of the gene and the characterization of the enzyme

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encoded by the gene with regard to its enzymatic properties.

MATERIALS AND METHODS

Strains and growth conditions *T. onnurineus* NA1 was isolated from a deep-sea hydrothermal vent area at the PACMANUS field (3°14′S, 151°42′E) in the East Manus Basin. YPS medium (15) was used to grow the strain for DNA manipulation. The culture and maintenance of the strain were performed using standard procedures (16). To prepare a seed culture of the strain, YPS medium in a 25-ml serum bottle was inoculated with a single colony formed on a phytagel plate, which gellan gum (Sigma, St. Louis, MO, USA) was used as a gelling agent, and then the colony was cultured at 85°C for 20 h. Seed cultures were used to inoculate 700 ml of YPS medium in an anaerobic jar, followed by incubation at 85°C for 20 h. *Escherichia coli* DH5α was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, La Jolla, CA, USA) and plasmid pET-24a(+) (Novagen, Madison, WI, USA) were used for gene expression. *E. coli* strains were cultured in Luria–Bertani (LB) medium containing the appropriate antibiotics at 37°C.

DNA manipulation and nucleotide sequencing DNA manipulations were performed using standard procedures, as described by Sambrook and Russell (16). Genomic DNA of *T. onnurineus* NA1 was isolated using standard procedures (17). Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI, USA). The small-scale preparation of plasmid DNA from *E. coli* cells was performed with a plasmid mini kit (Qiagen, Hilden, Germany). Nucleotide sequencing was performed with an automated sequencer (ABI3100) using a BigDye terminator kit (PE Applied Biosystems, Foster City, CA, USA).

Cloning and expression of deblocking aminopeptidase-encoding gene The full length deblocking aminopeptidase-encoding gene of *T. onnurineus* NA1 (*TNA1_DAP*) flanked by *Nde*I and *Xho*I sites was amplified by PCR using genomic DNA and two primers (sense [5′-CGACCCGG*CATATG*GTTGACTACGCGCTG CTCAAAAAG-3′] and antisense [5′-CTCCACAT*CTCGAG*GAG CTTAAGCCCCGGAATCTCTTC-3′]; the italicized sequences indicate the *Nde*I site in the sense primer and the *Xho*I site in the antisense primer). The amplified DNA fragments were digested with *Nde*I and *Xho*I and then ligated into *Nde*I/*Xho*I-digested pET-24a(+). The ligated fragments were transformed to *E. coli* DH5α. Candidates with the correct construct were selected by restriction enzyme digestion, and the nucleotide sequence of the clones was confirmed by sequencing. To express the recombinant DAP, an *E. coli* BL21-CodonPlus(DE3)-RIL strain transformed with the constructed plasmid was grown at 37°C in LB medium containing 50 μg/ml chloramphenicol and kanamycin to an optical density of 0.6 at 600 nm. Overexpression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) and incubating at 37°C for 3 h.

TNA1_DAP purification and analysis of bound metal ions The cells were harvested by centrifugation at 6000×*g* for 20 min and resuspended in 50 mM Tris–HCl buffer (pH 8.0), 0.1 M KCl and 10% glycerol. The cells were disrupted by sonication and centrifuged at 20,000×*g* for 1 h. The resulting supernatant was applied to a column containing TALON metal affinity resin (BD Biosciences Clontech, Palo Alto, CA, USA) and washed with 10 mM imidazole (Sigma) in 50 mM Tris–HCl buffer (pH 8.0), 0.1 M KCl and 10% glycerol, and TNA1_DAP was eluted with 300 mM imidazole in the buffer. The pooled fractions were then buffer-exchanged to 50 mM Tris–HCl buffer (pH 8.0) and 10% glycerol using Centricon YM-10 (Millipore, Bedford, MA, USA).

Protein concentration was estimated from the absorbance at 280 nm using the extinction coefficient 41,250 M^{-1} cm^{-1} (18). Protein purity was examined by SDS–PAGE performed according to the standard procedure. The bound metals of the purified protein were analyzed using Thermo Elemental X7 inductively coupled plasma mass spectrometer (ICP-MS; Thermo Elemental, Winsford, UK). TNA1_DAP purified by Superdex 200 10/300 GL column (Amersham Biosciences, Piscataway, NJ, USA) was applied to a HiTrap desalting column (Amersham Biosciences) equilibrated with 0.02% Chelex 100 (Bio-Rad Laboratories, Hercules, CA, USA)-treated 25 mM ammonium acetate. The desalted TNA1_DAP (4.6 mg/ml) was used for the analysis.

Enzyme assay TNA1_DAP activity was determined by analyzing the hydrolysis of Leu-*p*NA, Met-*p*NA and acetyl-leucine-*p*-nitroanilide (Ac-Leu-*p*NA) (Bachem AG, Bubendorf, Switzerland). The assay mixture (800 μl) contained 50 mM HEPES buffer (pH 7.5), 0.1 mM $CoCl_2$, 0.2 M NaCl and 2 mM substrate, and was preincubated at 80°C for 5 min. The reaction was initiated by adding the enzyme. The mixture was incubated at 80°C for 5 min, and the reaction was stopped by adding 1 M trichloroacetic acid (100 μl). The absorbance of the released *p*-nitroaniline was determined at 406 nm. The molar absorption coefficient for *p*-nitroaniline at 406 nm ($\varepsilon_{406\,nm}$=9910 M^{-1} cm^{-1}) was used for calculating enzyme activity.

Gel filtration chromatography TNA1_DAP was buffer-exchanged to 50 mM HEPES buffer (pH 7.5) using Centricon YM-10 (Millipore, Bedford, MA, USA) and then mixed with 0.2 M NaCl with or without 0.1 mM $CoCl_2$. The samples were analyzed on a Superdex 200 10/300 GL column using a fast protein liquid chromatograph (Äkta FPLC system; Amersham Biosciences), equilibrated with 50 mM HEPES (pH 7.5) and 0.2 M NaCl in the presence or absence of 0.1 mM $CoCl_2$. The column was calibrated with a sample from a molecular-mass standard kit (Sigma); the elution volumes for the standards fit well (R^2=0.99) when plotted against molecular mass.

TNA1_DAP autodegradation TNA1_DAP (4 μg) was incubated for 0.5 and 1 h at 80°C in 40 μl of 50 mM HEPES buffer (pH 7.5) and 0.1 mM $CoCl_2$, and was analyzed by SDS–PAGE (12%). The fragment formed on autodegradation was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Ultraflex; Bruker Daltonik, Bremen, Germany) and the identity of the fragment was inferred from the molecular mass of the peptide.

Nucleotide sequence accession number The nucleotide sequence data obtained has been submitted to the DDBJ/EMBL/GenBank databases under accession no. DQ862477.

RESULTS

Primary structure of *TNA1_DAP* and purification of recombinant protein Recently, we have isolated the hyperthermophilic archaeon *T. onnurineus* NA1, which grows at high temperatures (70–90°C). By analyzing its genome sequence, we found that an ORF composed of 1047 bp encodes a protein homologous to the deblocking aminopeptidases from *P. furiosus* DSM 3638 (86% identity) (5) and from *P. horikoshii* OT3 (35–83% identity) (6–7, 11) (Fig. 1). The gene was one of three deblocking aminopeptidase genes that are annotated in the genome database of *T. onnurineus* NA1 and exhibit over 75% identity to the three corresponding genes of DAPs from the *Pyrococcus* sp. (7). TNA1_DAP seemed to belong to M42 peptidase family in the MH clan of metallopeptidases because metal-binding residues (two histidines, two aspartates, and one glutamate) and catalytic residues (one aspartate and one glutamate) are well conserved. *TNA1_DAP* was amplified by PCR, and the expressed enzyme was purified from a soluble cell extract.