# Cloning, Expression, and Characterization of Aminopeptidase P from the Hyperthermophilic Archaeon *Thermococcus* sp. Strain NA1

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Genomic analysis of a hyperthermophilic archaeon, *Thermococcus* sp. strain NA1, revealed the presence of a 1,068-bp open reading frame encoding a protein consisting of 356 amino acids with a calculated molecular mass of 39,714 Da (GenBank accession no. DQ144132). Sequence analysis showed that it was similar to the putative aminopeptidase P (APP) of *Thermococcus kodakaraensis* KOD1. Amino acid residues important for catalytic activity and the metal binding ligands conserved in bacterial, nematode, insect, and mammalian APPs were also conserved in the *Thermococcus* sp. strain NA1 APP. The archaeal APP, designated TNA1_APP (*Thermococcus* sp. strain NA1 APP), was cloned and expressed in *Escherichia coli*. The recombinant enzyme hydrolyzed the amino-terminal Xaa-Pro bond of Lys($N^\varepsilon$-Abz)-Pro-Pro-pNA and the dipeptide Met-Pro ($K_m$, 0.96 mM), revealing its functional identity. Further enzyme characterization showed the enzyme to be a $Co^{2+}$-, $Mn^{2+}$-, or $Zn^{2+}$-dependent metallopeptidase. Optimal APP activity with Met-Pro as the substrate occurred at pH 5 and a temperature of 100°C. The APP was thermostable, with a half-life of >100 min at 80°C. This study represents the first characterization of a hyperthermophilic archaeon APP.

Aminopeptidase P (APP, or X-Pro aminopeptidase; EC 3.4.11.9) is a peptidase that specifically removes the N-terminal amino acids from peptides in which the penultimate residue is proline (5). Since the time an enzyme with the specificity of APP was first purified from *Escherichia coli* (24), APPs have been characterized from diverse sources, including bacteria (16), nematodes (15), insects (14), plants (10), and tissues from several mammalian species (9, 11). While the physiological role of APP in bacteria is unclear, mammalian APP is involved in the protein turnover of collagen and the regulation of biologically active peptides, such as substance P and bradykinin (5, 23, 26). It has been shown that APPs from a number of lactococcal strains may contribute to the abolition of bitterness during the ripening of cheese by participating in peptide degradation following release into the cheese matrix (17).

To date, however, there have been no reports on the properties of an APP from either an archaeon or a hyperthermophile. With the availability of a generally applicable combination of conventional genetic engineering and genomic research techniques, the genome sequences of some hyperthermophilic microorganisms are of considerable biotechnological interest because of their heat-stable enzymes, and many extremely thermostable enzymes are being developed for biotechnological purposes (22). Furthermore, recent advances in the application of molecular biological tools to hyperthermophilic archaea, such as gene knockout techniques and efficient transformation systems, could facilitate the study of hyperthermophilic archaeal gene function and contribute to an understanding of the physiology of hyperthermophilic archaea.

To facilitate the search for valuable and extremely thermostable enzymes and to help answer questions concerning the physiology of hyperthermophilic archaea grown at extremely high temperatures, we recently isolated a hyperthermophilic archaeon, *Thermococcus* sp. strain NA1 (S. S. Bae et al., unpublished data), and its whole genome sequence was determined (J.-H. Lee et al., unpublished data). Analysis of the genome information of *Thermococcus* sp. strain NA1 revealed an APP gene that is similar to those for other APPs. In the present study, the gene corresponding to an APP was cloned and expressed in *E. coli*. The recombinant enzyme was purified, and its characteristics were examined.

## MATERIALS AND METHODS

**Strains and growth conditions.** *E. coli* DH5α was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, La Jolla, CA) and plasmid pET-24a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani medium at 37°C, and kanamycin was added to the medium at a final concentration of 50 μg/ml.

**DNA manipulation and sequencing.** DNA manipulations were performed by standard procedures as described by Sambrook and Russell (19). Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI). Small-scale preparations of plasmid DNA from *E. coli* cells were performed with a plasmid mini kit (QIAGEN, Hilden, Germany). DNA sequencing was performed with an automated sequencer (ABI 3100), using a BigDye Terminator kit (PE Applied Biosystems, Foster City, CA).

**Cloning and expression of APP-encoding gene.** The full-length *Thermococcus* sp. strain NA1 APP gene, flanked by NdeI and XhoI sites, was amplified from genomic DNA by PCR using the following two primers: sense, 5′-CGACCC *GGCATATG*CGCCTCAACAAGCTCACTTCTCTG-3′; and antisense, 5′-CTC CACAT*CTCGAG*CACGATTATCAGCTCCCTCGGTGCC-3′. The italicized sequences indicate the NdeI site in the sense primer and the XhoI site in the antisense primer. The amplified DNA fragment was digested with NdeI and XhoI, the fragment was ligated with NdeI/XhoI-digested plasmid pET-24a(+), and the resultant recombinant plasmid was used to transform *E. coli* DH5α. The recombinant plasmid was introduced into BL21-CodonPlus(DE3)-RIL cells for expression after sequence confirmation. Overexpression was induced by the

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TNA1APP    ----------MRLNKLTSLLKEKAFDGALISPGTNLYYLTSLHIHEAGERLTVLAVNSNG  50
EcAPP      MSEISRQEFQRRRQALVEQMQPGSAALIFAAPEVTRSADSEYPYRQNSDFWYFTGFNEPE  60
PfProl     --------MKERLEKLVKFMDENSIDRVFIAKPVNVYYFSGTSPLGGGY----IIVDGD-  47
EcMetAP    ----------------------------------------------------------
                   .   . :       :    .   :      .        .

TNA1APP    EYHLLAP-------SLYENVIRDFPATFWRDG-------------------ENPYGKLAE  84
EcAPP      AVLVLIKSDDTHNHSVLFNRVRDLTAEIWFGRRLGQDAAPEKLGVDRALAFSEINQQLYQ 120
PfProl     EATLYVP-------ELEYEMAKEE-SKLPVVK------------------FKKFDEIYE  80
EcMetAP    ----------------------------------------------------------
                    .   .   . .:                        .   .   .

TNA1APP    ILKELDLS---SGRILVENTMRADWLIGILKLGSFEFHP------LSILMRELRMRKDAE 135
EcAPP      LLNGLDVVYHAQGEYAYADVIVNSALEKLRKGSRQNLTAPATMIDWRPVVHEMRLFKSPE 180
PfProl     ILKNTETL  GIEGTLSYSMVENFKE  KSNVKEFKK    IDDVIKDLRIIKTKE 128
EcMetAP    --------------------------------------------------MAISIKTPE   9
                .  .                 .      .  . .         * *

TNA1APP    EIRLMEKAAKIVDEVFEEIIGMDLIGMREKDLALKIELLIRER-------SDGISFEPIV 188
EcAPP      EIAVLRRAGEITAMAHTRAMEKCRPGMFEYHLEGEIHHEFNRHG------ARYPSYNTIV 234
PfProl     EIEIIEKACEIADKAVMAAIEEITEGKREREVAAKVEYLMKMNG------AEKPAFDTII 182
EcMetAP    DIEKMRVAGRLAAEVLEMIEPYVKPGVSTGELDRTCNDYIVNEQHAVSACLGYHGYPKSV  69
                :*   :. * .:.  .          *     .:    .  . :.*  ::  .    .: :

TNA1APP    ASGENGANPHHAPGERKLG-EGDLVILDYGAKWEGYCSDITRTIALG-KPDKKLLEIYEV 246
EcAPP      GSGENGCILHYTENECEMR DGDLVLIDAGCEYKGYAGDITRTFPVNGKFTQAQREIYDI 293
PfProl     ASGHRSALPHGVASDKRIE-RGDLVVIDLGALYNHYNSDITRTIVVG-SPNEKQREIYEI 240
EcMetAP    CISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVG KPTIMGERLCRI 128
                .  .    *     .:  .:    **:* :*    . : .*  ::  ::  .    .:  :

TNA1APP    VKNAQEGAFQTVREGLKAKEVDKAAR------------------NYIAEAGYGEYFTHRT 288
EcAPP      VLESLETSLRLYRPGTSILEVTGEVVKIMVSGLVKLGILKGDVDELIAQNAHRPFFMHGL 353
PfProl     VLEAQKRAVEAAKPGMTAKELDSIAR------------------EIIKEYGYGDYFIHSL 282
EcMetAP    TQESLYLALRMVKPGINLREIGAAIQ------------------KFVEAEGFS-VVREYC 169
                . ::    :..   : * .  *:                   : :   ..   .   :

TNA1APP    GHGLGLDVIIEEPYIGPDGE----VILKNGMTFTIEPGIYVP---------------GL 327
EcAPP      SHWLGLDVHDVGVYGQDRS----RILEPGMVLTVEPGLYIAPDAEVPEQY--------RG 401
PfProl     GHGVGLEIIEWPRISQYDE----TVLKEGMVITIEPGIYIP---------------KL 321
EcMetAP    GHGIGRGFHEEPQVLHYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRS 229
                .* :*   *:.        :*: **.:*:** :

TNA1APP    GGVRIEDDVVVEGGKGRRLTKAPR------ELIIV------  356
EcAPP      IGIRIEDDIVITETGNENLTASVVKKPEEIEALMVAARKQ-  441
PfProl     GGVRIEDTVLITENGAKRLTKTER------ELL--------  348
EcMetAP    LSAQYEHTIVVTDNGCEILTLRKD------DTIPAIISHDE  264
                . : *. :::     . **      : :
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FIG. 1. Sequence comparison of *Thermococcus* sp. strain NA1 aminopeptidase P (TNA1APP), *E. coli* aminopeptidase P (EcAPP; gi:113751), *P. furiosus* prolidase (PfProl; gi:17380168), and *E. coli* methionine aminopeptidase (EcMetAP; gi:113740). Dashes indicate gaps, and numbers on the right represent the positions of the last residues in the original sequence. Identical residues among the four enzymes are marked with asterisks, and residues with conserved substitutions and semiconserved substitutions are marked with colons and dots, respectively. The putative active-site residues participating in metal ion coordination and proton shuffling are shown in bold and are underlined, respectively.

addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase, followed by incubation for 3 h at 37°C. The cells were harvested by centrifugation (6,000 × $g$, 20 min, 4°C) and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol. The cells were disrupted by sonication and centrifuged (20,000 × $g$, 1 h, 4°C). The resulting supernatant was applied to a column of TALON metal-affinity resin (BD Biosciences Clontech, Palo Alto, CA) and washed with 10 mM imidazole (Sigma, St. Louis, MO) in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol, and APP was eluted with 300 mM imidazole in the buffer. The pooled fractions were then buffer exchanged with 50 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol, using a Centricon YM-10 column (Millipore, Bedford, MA).

The protein concentration was estimated from the absorbance at 280 nm, using an extinction coefficient of 33,710 $M^{-1}$ $cm^{-1}$. The protein purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to a standard procedure.

**Enzyme assays.** APP-catalyzed hydrolysis of L-lysyl(ε-2-aminobenzoyl)-L-prolyl-L-prolyl-4-nitroanilide [Lys($N^ε$-Abz)-Pro-Pro-pNA; Bachem AG, Bubendorf, Switzerland] was detected by the release of Lys($N^ε$-Abz)-OH, which was monitored at an excitation wavelength of 320 nm and an emission wavelength of

417 nm at 80°C (21), using an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The reaction mixture (1 ml) consisted of 50 mM sodium acetate buffer (pH 5.0), 15 μM Lys($N^ε$-Abz)-Pro-Pro-pNA, and 1.2 mM CoCl$_2$. The reaction was initiated by adding 2 μg APP. In all routine assays, APP activity was measured by detecting proline liberated by the hydrolysis of Met-Pro (Bachem AG) which was dissolved in methanol. The proline concentration was determined by a modification of the colorimetric ninhydrin method of Yaron and Mlynar (25). The ninhydrin reagent was prepared by the addition of 3% (wt/vol) ninhydrin (Sigma) to a mixture of 60% (vol/vol) glacial acetic acid and 40% (vol/vol) phosphoric acid, followed by a 30-min incubation at 70°C. The assay mixture for APP (300 μl), which contained 50 mM sodium acetate buffer (pH 5.0), 4 mM Met-Pro, and 1.2 mM CoCl$_2$, was incubated at 80°C for 5 min. The reaction was initiated by the addition of the enzyme, the mixture was incubated at 80°C for a further 5 min, and the reaction was stopped by the addition of glacial acetic acid (300 μl) followed by the ninhydrin reagent (300 μl). After being heated at 80°C for 10 min, the solution was cooled on ice, and the absorption at 515 nm was determined. The amount of released proline was calculated, using an extinction coefficient of 4,570 $M^{-1}$ $cm^{-1}$ for the ninhydrin-proline complex. One unit of APP activity was defined as the

amount of enzyme that liberated 1 μmol proline min$^{-1}$ under these assay conditions.

## RESULTS

**Primary structure of APP gene and expression of recombinant enzyme.** Recently, we isolated a hyperthermophilic archaeon, *Thermococcus* sp. strain NA1, grown at high temperatures of 70 to 90°C. By analyzing the genome sequence, we found that an open reading frame (ORF) composed of 1,068 bp encodes a protein consisting of 356 amino acids with a predicted molecular mass of 39,714 Da. Database searches indicated that the amino acid sequence of TNA1_APP showed significant similarity to those encoded by the putative APP genes in the genome sequences of *Thermococcus kodakaraensis* KOD1 (75% identity) (6) and *Pyrococcus abyssi* GE5 (74% identity) (3) as well as to that of the dipeptidase from *Pyrococcus horikoshii* OT3 (74% identity) (13). Compared with all the known APPs or prolidases, that is, *E. coli* APP (26%), the cytosolic APPs of *Drosophila melanogaster* (17%), humans (19%), rats (21%), tomato plants (14%), and *Caenorhabditis elegans* (20%), *E. coli* prolidase (20%), and archaeon *Pyrococcus furiosus* prolidase (36%), TNA1_APP yielded low amino acid sequence identities of below 36% (7). Amino acid sequence analysis revealed that five divalent metal ligands (two aspartic acid residues, a histidine residue, and two glutamic acid residues) and putative proton shuttle sites (three histidine residues), all originally identified in the crystal structure of *E. coli* APP (24), were highly conserved in TNA1_APP (Fig. 1). Based on this analysis, it seemed likely that the ORF is a member of a "pita bread-fold" family (1). A Clustal W alignment of the amino acid sequences of TNA1_APP, *E. coli* APP, *P. furiosus* prolidase, and *E. coli* methionine aminopeptidase is shown in Fig. 1. Because prolidase (proline dipeptidase; EC 3.4.13.9) only cleaves dipeptides with proline at the C terminus (5), it was necessary to confirm whether TNA1_APP was capable of hydrolyzing oligopeptides longer than dipeptides to classify the enzyme as aminopeptidase P.

To address this, the APP gene was amplified by PCR, and the expressed enzyme was purified from a soluble cell extract as described in Materials and Methods. An analysis by SDS-PAGE showed that a 41-kDa protein (Fig. 2), which was the expected size of the fusion product comprising the 40-kDa APP protein and a 1-kDa peptide corresponding to -LEH$_6$- (His tag) at the C terminus, was the major component of the purified sample.

**Biochemical enzyme characterization.** The purified recombinant protein was tested for activity toward a typical APP substrate, and it displayed APP activity by hydrolyzing Lys($N^\varepsilon$-Abz)-Pro-Pro-pNA (data not shown). APP is a peptidase that specifically removes the N-terminal amino acids from peptides in which the penultimate residue is proline (5), although in a number of cases it is unable to hydrolyze a dipeptide with proline in the C-terminal position (16, 17, 27). To show whether the recombinant enzyme was capable of hydrolyzing a dipeptide as well as Lys($N^\varepsilon$-Abz)-Pro-Pro-pNA, the hydrolyzing activity toward Met-Pro was also tested, and the enzyme was clearly able to hydrolyze the Met-Pro dipeptide. A kinetic analysis was conducted using Met-Pro, and kinetic parameters such as $K_m$ (0.96 mM) and $k_{cat}$ (541 s$^{-1}$) were calculated from the measured activity.
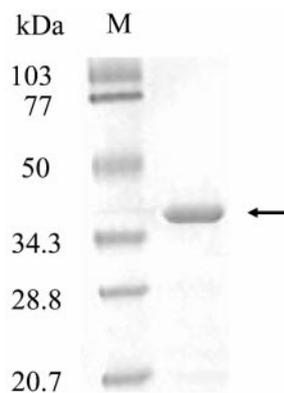


FIG. 2. SDS-PAGE (12%) of the purified enzyme. The molecular mass standards (lane M) were phosphorylase *b* (103 kDa), bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), soybean trypsin inhibitor (28.8 kDa), and lysozyme (20.7 kDa). The band corresponding to the enzyme is indicated by the arrow.

As shown in Fig. 3A, APP activity toward Met-Pro was strongly stimulated at high temperatures and showed a temperature optimum of ≥100°C, with <10% of maximal activity observed at 40 to 50°C. The same pattern was shown with Lys($N^\varepsilon$-Abz)-Pro-Pro-pNA. The influence of pH on APP activity was evaluated by using different buffers in the pH range of 4 to 8. Interestingly, the optimum pHs of APP activity were different for Met-Pro (Fig. 3B) and Lys($N^\varepsilon$-Abz)-Pro-Pro-pNA (Fig. 3C), with values of 5 and 6.5 to 7, respectively. The reason that the two substrates could be hydrolyzed at different pH values needs to be understood, and further analysis is under way.

The enzyme's thermostability was evaluated by incubating the enzyme in 50 mM sodium acetate buffer, pH 5, at 80 and 90°C. APP was very thermostable, losing enzymatic activity with half-life values ($t_{1/2}$) of >100 min at 80° and 49 min at 90°C (Fig. 4). Interestingly, incubating APP at 80°C appeared to increase the relative activity up to 20% within 20 min.

The presence of the conserved amino acid residues for metal binding implied that TNA1_APP would be influenced by the addition of metal ions. To examine the metal ion requirement of TNA1_APP, endogenous metal ions were removed by ultrafiltration against metal-free Tris-HCl buffer. After incubation of TNA1_APP with various metal ions (1.2 mM), the enzyme activity increased in the presence of Co$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ ions, although a little or no activation was observed with other divalent cations, such as Ba$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, and Ni$^{2+}$. The effects of Co$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ concentrations on the activity of APP were very different, with maximal activity at 3 mM CoCl$_2$, 20 mM MnCl$_2$, and 0.4 mM ZnCl$_2$ (Fig. 5).

## DISCUSSION

This study is believed to be the first to characterize an APP from either an archaeon or a hyperthermophile. The APP gene has been predicted by sequence analysis, and its function was confirmed by biochemical characterization of the recombinant enzyme expressed in *E. coli*.

The kinetic parameters calculated by kinetic analysis using Met-Pro, i.e., $K_m$ (0.96 mM) and $k_{cat}$ (541 s$^{-1}$), showed that the dipeptide was a competent substrate of TNA1_APP and were
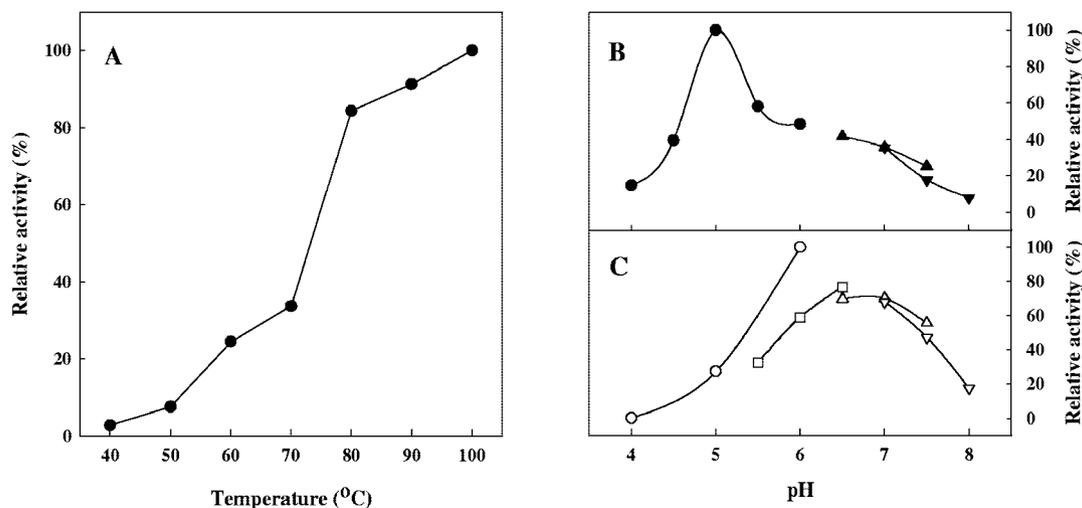
FIG. 3. Effects of temperature (A) and pH (B and C) on activity of APP. (A) Activity assays were performed under standard conditions as the sample temperature was increased from 40 to 100°C. (B and C) Activity assays were performed under standard conditions for the hydrolysis of Lys($N^{\varepsilon}$-Abz)-Pro-Pro-pNA (open symbols) and Met-Pro (closed symbols) with the following buffers (each at 50 mM): sodium acetate (circles), pH 4 to 6; KMES [potassium 2-(N-morpholino)ethanesulfonic acid] (squares), pH 5.5 to 7; MOPS (morpholinepropanesulfonic acid) (triangles), pH 6.5 to 7.5; and HEPES (upside-down triangles), pH 7 to 8.

comparable to those for the recombinant form of *P. furiosus* prolidase obtained using the same dipeptide ($K_m$, 3.3 mM; $k_{cat}$, 525 s$^{-1}$) (7). However, whereas TNA1_APP was capable of hydrolyzing tripeptides such as Met-Pro-Gly (data not shown) and Lys($N^{\varepsilon}$-Abz)-Pro-Pro-pNA, it is known that *P. furiosus* prolidase does not hydrolyze tri- or tetrapeptides (7).

All other APPs from mesophilic sources show optimum activities at temperatures up to 55°C (4, 14, 15, 28). Considering that most proteases and peptidases isolated from *Thermococcus* spp. have enhanced activities at moderately high temperatures

of 70 to 85°C, except for a thermostable thiol protease purified from the extracellular fraction of *T. kodakaraensis* KOD1 (18), it is intriguing that TNA1_APP showed an optimum temperature above 100°C. The fact that TNA1_APP is a hyperthermostable enzyme was also demonstrated by the enzyme having a $t_{1/2}$ of >100 min at 80°C. Furthermore, TNA1_APP seemed to be significantly activated by incubation at high temperatures. Heat-induced activation over a short period of time has also been observed with other proteolytic enzymes purified from *P. horikoshii*, *Sulfolobus solfataricus*, and *Thermococcus* sp. strain NA1, including an acylamino acid-releasing enzyme (12), intracellular protease (8), prolyl endopeptidase, and carboxy-
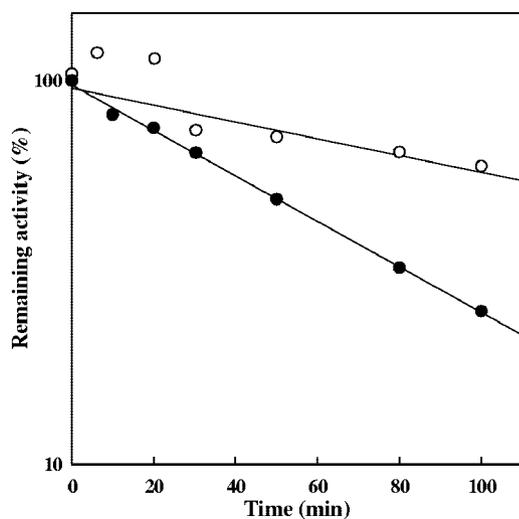


FIG. 4. Thermal inactivation of APP. Semilog plots of the remaining activity versus the incubation time are shown. APP (4 nM) was incubated at 80°C (○) or 90°C (●) in 50 mM sodium acetate buffer, pH 5, containing 1.2 mM CoCl₂. At the times shown, aliquots were removed, and the activity was measured in the same buffer at 80°C, using Met-Pro as the substrate. The lines were obtained by linear regression of the data.
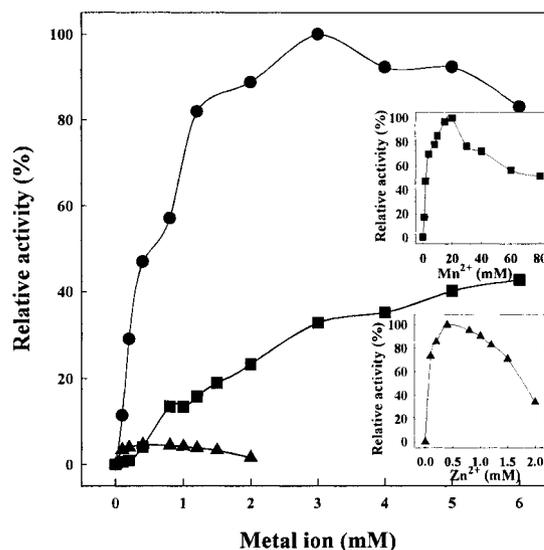


FIG. 5. Effects of metal ions on APP activity. Activity assays were performed under standard conditions while the concentration of divalent metal ions was varied. ●, Co$^{2+}$; ■, Mn$^{2+}$; ▲, Zn$^{2+}$.

peptidase (Lee et al., submitted for publication). Although the mechanism was not investigated, it can be deduced that a heat-induced conformational change in the enzyme increased its activity. Guagliardi et al. (8) are of the view that the stimulation of activity upon preheating is a feature of some enzymes from thermophilic sources, and they believe that this reflects the activation of the low-activity status adopted by these catalysts at moderate temperatures.

The pH optimum of TNA1_APP was in a mildly acidic range (pH 5 and 6.5 to 7), which is distinct from other APPs. Most APPs show optimum activity at pH 7 to 9, although a membrane-bound APP from a bovine lung had a pH optimum of 6.5 for bradykinin hydrolysis (20). Furthermore, the optimum pH values were different for the hydrolysis of Met-Pro and Lys($N^\varepsilon$-Abz)-Pro-Pro-pNA, and the reasons for this need to be investigated.

The APP from *Thermococcus* sp. strain NA1 appeared to be a metalloenzyme stimulated by $Co^{2+}$ and $Mn^{2+}$, like the enzyme from *Lactococcus lactis* (16, 17) and those from other sources (2, 11). $Zn^{2+}$ also showed a stimulatory effect, as opposed to the $Zn^{2+}$ inhibition of all mammalian and *C. elegans* APPs.

We have been able to establish the function of a predicted ORF in a hyperthermophilic archaeon by combining conventional genetic engineering and genome research techniques. The hyperthermophilic archaea whose sequences have been determined in the *Thermococcaceae* family have genomes consisting of about 2,000 genes, and almost half of the predicted ORFs are putative or hypothetical (6). Currently, studies on the physiology and genetics of hyperthermophiles are limited by a lack of molecular biological tools and by the extreme growth conditions required. We hope that a genome-wide approach of the kind used in the present study can contribute to establishing the function of putative or hypothetical genes, eventually leading to an understanding of archaeal physiology.

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