Acylamino Acid-releasing Enzyme from the Thermophilic Archaeon Pyrococcus horikoshii*

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When the genome of the thermophilic archaeon Pyrococcus horikoshii was sequenced, a gene homologous to the mammalian gene for an acylamino acid-releasing enzyme (EC 3.4.19.1) was found in which the enzyme's proposed active residues were conserved. The P. horikoshii gene comprised an open reading frame of 1,896 base pairs with an ATG initiation codon and a TAG termination codon, encoding a 72,390-Da protein of 632 amino acid residues. This gene was overexpressed in Escherichia coli with the pET vector system, and the resulting enzyme showed the anticipated amino-terminal sequence and high hydrolytic activity for acylpeptides. This enzyme was concluded to be the first acylamino acid-releasing enzyme from an organism other than a eukaryotic cell. The existence of the enzyme in archaea suggests that the mechanisms of protein degradation or initiation of protein synthesis or both in archaea may be similar to those in eukaryotes. The enzyme was stable at 90 °C, with its optimum temperature over 90 °C. The specific activity of the enzyme increased 7-14-fold with heat treatment, suggesting the modification of the enzyme's structure for optimal hydrolytic activity by heating. This enzyme is expected to be useful for the removal of N\(^{\text{acetyl}}\)-acylated residues in short peptide sequence analysis at high temperatures.

The acylamino acid-releasing enzyme (AARE) catalyzes the NH\(_{2}\)-terminal hydrolysis of N\(^{\text{acetyl}}\)-acylated amino acids (1). AARE has been used for removal of N\(^{\text{acetyl}}\)-acylated residues in protein sequence analysis. Until now, AARE has been isolated only from eukaryotic cells (1-4) and characterized as its own serine protease subfamily (5, 6). The physiological role of the enzyme is not clear, although it has been suggested that it affects the processing or sorting of proteins (7, 8) in eukaryotic cells. From eukaryotic cells, some AARE genes have already been cloned (9, 10). However, the production and expression of AARE from these genes within Escherichia coli have not been carried out.

Pyrococcus horikoshii (OT3) is one of the thermophilic archaea collected from a volcanic vent in the Okinawa trough (11). The optimum growth temperature of this archaeon ranges from 90 to 105 °C. Most of the proteins from P. horikoshii are thought to be thermostable and active at high temperature. The size of its genome is about 2 Mb, and the guanine-cytosine content is relatively low. At the National Institute of Technology and Evaluation (Tokyo, Japan), sequencing of this genome is in progress (11). From the genome sequencing in P. horikoshii we found a gene that had some homology with a gene for AARE from pig liver (9, 12). Therefore, we cloned the gene from P. horikoshii and attempted to express the enzyme in E. coli and examine the characteristics of the expressed enzyme.

EXPERIMENTAL PROCEDURES

Materials—The host E. coli BL21(DE3) and the vector pET11a were obtained from Novagen (Madison, WI). The Pfu DNA polymerase, restriction enzymes, and ligation kit were purchased from Takara Shuzo (Otsu, Shiga, Japan). The N\(^{\text{acetyl}}\)-acylamino acid p-nitroanilide derivatives (Ac-amino acid-pNA) were purchased from Sigma (St. Louis, MO) and Bachem (Bubendorf, Switzerland). The acylpeptides Ac-Ala-Ala, Ac-Met-Ala, f-Met-Ala, f-Met-Ala-Ser, f-Met-Leu-Gly, and f-Ala-Ala-Ala were also from Bachem. The other acylpeptides Ac-Met-Ala-Ala-Ala-Ala, Ac-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala, and f-Met-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ala were purchased from Peptide Institute Inc. (Minou, Osaka, Japan). o-Melanoctye-stimulating hormone (o-MSH) was purchased from Funakoshi (Tokyo, Japan). The synthesis of DNA primers and the sequencing of proteins were performed by the custom service center of Takara Shuzo. The DNA sequencing was carried out with ABI model 373 sequencer (Perkin-Elmer, Applied Biosystems Div., Foster City, CA). All other chemicals were of the highest reagent grade commercially available.

Cloning and Expression of the Gene—The genome of P. horikoshii was sequenced by the method of Kaneko et al. (13). The gene that was homologous to the mammalian gene for AARE was found by BLAST search (14). The gene was amplified by the polymerase chain reaction method using two primers with unique restriction sites. Amplification of the gene by polymerase chain reaction was carried out at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, for 35 cycles using Pfu DNA polymerase. The amplified gene was hydrolyzed by the restriction enzymes and inserted in pET11a cut by the same restriction enzymes. The amplified gene was expressed using the pET11a vector system in the host E. coli BL21(DE3) according to the manufacturer’s instructions. The host E. coli BL21(DE3) was transformed by the constructed plasmid. The transformant cell was grown in 2YT medium (1% yeast extract, 1.6% tryptone, and 0.5% NaCl) containing ampicillin (100 µg/ml) at 37 °C. After incubation with shaking at 37 °C until the A\(_{600}\) reached 0.6-1.0, the induction was carried out by adding isopropyl β-D-thiogalactopyranoside at a final concentration of 1 mM and shaking for 4 h at 37 °C. The concentration of the enzyme was determined with Coomassie protein assay reagent (Pierce Chemical Company, Rockford, IL) using bovine serum albumin as the standard protein.

Purification of the Enzyme—After induction, the transformant cells were harvested by centrifugation and disrupted with oxide aluminum in 50 mM Tris-HCl buffer (pH 8.0) containing 0.6 M NaCl. After incubation with DNase I (from bovine pancreas; Sigma) for 30 min at room temperature, the enzyme was purified by the method of Kaneko et al. (13). The enzyme was purified by a combination of gel filtration, ion-exchange chromatography, and size exclusion chromatography. The enzyme was stable at 90 °C, with its optimum temperature over 90 °C. The specific activity of the enzyme increased 7-14-fold with heat treatment, suggesting the modification of the enzyme's structure for optimal hydrolytic activity by heating. This enzyme is expected to be useful for the removal of N\(^{\text{acetyl}}\)-acylated residues in short peptide sequence analysis at high temperatures.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB009494.‡ To whom correspondence should be addressed. Fax: 81-298-54-6151; E-mail: ishikawa@nihh.go.jp.

‡ The abbreviations used are: AARE, acylamino acid-releasing enzyme; Ac-, N\(^{\text{acetyl}}\)-acyl; f-, N\(^{\text{formyl}}\)-formyl; pNA, p-nitroanilide; o-MSH, o-melanoctye-stimulating hormone; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; DMF, N, N-dimethylformamide; DSC, differential scanning calorimetry; AAREP, AARE from P. horikoshii; HAAREP, heat-activated AAREP.
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temperature, the crude extract was heated at 85 °C for 30 min. The supernatant obtained by centrifugation was dialyzed against 50 mM Tris-HCl buffer (pH 8.0). The dialyzed sample was loaded on a HiTrap Q column (Pharmacia, Uppsala, Sweden). The column was washed with 50 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient (0.0 to 0.6 M NaCl) in the same buffer. The fractions that showed proteolytic activity of a similar molecular mass (70 kDa, SDS-PAGE) calculated from the amino acid sequence were concentrated by a Centricon 10 filter (Amicon Inc., Beverly, MA). The concentrated material was loaded on a HiLoad Superdex 200 column (Pharmacia) and eluted with 100 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM NaCl. The fractions demonstrating only one protein band with a molecular mass of 70 kDa by SDS-PAGE were collected and used for the detailed characterizations of the enzyme.

**Molecular Weight Determination**—The molecular weight of the enzyme was determined by SDS-PAGE performed on a 4–15% gradient gel in the Phast System (Pharmacia). Protein bands were visualized by staining with Coomassie Brilliant Blue.

The molecular weight was also determined by high performance liquid chromatography (HPLC) and light-scattering photometry. The HPLC was performed on a Superdex 200 column (Pharmacia), and the elution was carried out using 100 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM NaCl at 1.5 ml/min at room temperature. The eluted protein was detected by its absorbance at 280 nm. The light-scattering photometer was conducted at room temperature with a DLS-700S light-scattering photometer (Shimadzu, Japan) calibrated with benzene (15) at 633 nm and analyzed by the method of Kamata and Nakahara (16). Optical clarification was performed with polyvinylidene fluoride filters. The specific refractive index increment (dn/dc) was determined by analysis of more than 80 archaeal promoters

The experiments of DSC were performed in a model DSC5100 calorimeter (TA Instruments, New Castle, DE) at 2.5 °C/min, heating rate. The specific heat capacity was calculated using the empirical method of Searle and co-workers (17). The data were fitted with a polynomial function for the baseline, and the heat capacity was determined. The protein sample was prepared by dissolving the protein in 1.0 M NaCl at 1.5 ml/min at room temperature. The eluted protein was detected by its absorbance at 280 nm. The light-scattering photometer was conducted at room temperature with a DLS-700S light-scattering photometer (Shimadzu, Japan) calibrated with benzene (15) at 633 nm and analyzed by the method of Kamata and Nakahara (16). Optical clarification was performed with polyvinylidene fluoride filters. The specific refractive index increment (dn/dc) was determined by analysis of more than 80 archaeal promoters

The gene was amplified by polymerase chain reaction using two primers. The upper primer (5'-TTTTGATCTCTATAT-GGGCAAGGGCGTTTCA-3') contained an NdeI site (underlined), and the lower primer (5'-TTTTGTATACCTTGGATCC TAAGGGTTAGCTATCCTTT-3') contained a BamHI site (underlined). The amplified gene was inserted in pET11a, and BL21(DE3) was transformed by the constructed plasmid. After induction for 4 h at 37 °C, 50 mg of the thermostable 70-kDa protein (as determined by SDS-PAGE) was purified from 2 liters of culture medium. The result of densitometer (data not shown) for SDS-PAGE (Fig. 2) indicated that the purity was about 99%. The purified protein (0.05 mg) in solution was spotted on an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and sequenced by a PSQ-1 protein sequencer (Shimazu) at the custom service center of Takara Shuzo. By sequence analysis, the first 20 amino acid residues of the NH2 terminus except the NH2-terminal Met were detected. The NH2-terminal sequence was identical to that anticipated from the nucleotide sequence. The extra f-Met residue at the NH2 terminus of the nascent polypeptide, encoded by the initiation codon, was not detected. This shows that the Gly residue neighboring the starting f-Met residue has a small radius of gyration which is essential for the removal of the f-Met residue to yield the mature enzyme (20), and the soluble protein was processed correctly. The high yield of the recombinant protein indicates the very efficient post-translation of the P. horikoshii gene inside E. coli cells, including the removal of the f-Met residue from the nascent polypeptide. It is suggested that the pET system is a good tool for the production of this protein, and the protein has no toxic effect on the growth of E. coli.

Unlike other AARE, the protein derived from P. horikoshii needed a high concentration of NaCl to be dissolved. Therefore, the purified protein solution used for the characterization contained 0.6 M NaCl. The molecular mass of the purified protein, as determined by SDS-PAGE (Fig. 2), was consistent with that (72,390 Da) calculated from the amino acid sequences. The molecular mass of the protein determined by HPLC was about 150,000 Da (data not shown). The weight-averaged molecular weight was measured by light-scattering photometry using the dn/dc value determined for chicken egg white lysozyme at 158,000. Therefore, the protein is likely a dimer structure, instead of the four identical subunits found in mammals (9, 17). The absorption coefficient (A280 nm) of the protein at 1% was determined to be 12.0.

**Specificity of the Enzyme**—To examine the activity of this protein, we used Ac-Leu-pNA, Ac-Ala-pNA, Ac-Tyr-pNA, Leu-pNA, and Ala-pNA as substrates. Table I shows the hydrolytic activity (releasing of pNA) of the protein for them. At 85 °C and pH 5.4, the protein exhibited some hydrolytic activity for Ac-Leu-pNA, Ac-Ala-pNA, Ac-Tyr-pNA and no hydrolytic activity for Leu-pNA and Ala-pNA. As shown in Table I, the protein also had hydrolytic activity for acetylpeptides and formylpeptides. The analysis of the products by HPLC revealed that the protein could only release the acylated amino acids from acetylpeptides. Therefore, this protein was concluded to be the AARE from the thermophilic archaeon P. horikoshii. The char-
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characteristics of this enzyme (hereafter referred to as AAREP) were examined. The optimum pH of AAREP at 85 °C was between pH 4.8 and 5.5 (Fig. 3). The optimum temperature of AAREP at pH 5.4 was about 90 °C (Fig. 4). Its specificity for small substrates was different from those of AAREs in mammals (1, 17). Unlike the AARE from rat, AAREP released Ac-Leu better than Ac-Ala from Ac-amino acid-pNA; for most of the substrates used, the specific activity of AAREP was higher than that of AARE from rat (Table I). The activity decreased with increasing the residues of acylpeptides (Table I). Table II shows that AAREP has similar binding affinity for Ac-Leu-pNA, Ac-Ala-pNA, Ac-Ala-Ala, and Ac-Ala-Ala-Ala-Ala. This result is different from that of rat (17). The Km value obtained for Ac-Ala-Ala was a little smaller than that for Ac-Ala-Ala-Ala-Ala (Table II). The active site of AAREP seems to be suitable for relatively short acylpeptides. The hydrolytic activity of AAREP toward α-MSH was also examined under the above conditions. The NH2-terminal amino acid sequence of α-MSH was not detected by the protein sequencer after the incubation with AAREP. This result indicates that AAREP cannot release Ac-Ser from α-MSH, unlike the AARE of pig2 or rat (17). It is speculated that AAREP is able to hydrolyze only short acylpeptides.

Thermostability of the Enzyme—Thermostability of the enzyme was examined with CD and DSC. The CD spectrum in the far-UV region of the enzyme was examined at 25 °C and 95 °C. The CD spectrum of the enzyme at 95 °C was a little different from that at 25 °C (Fig. 5). The intensity of the negative ellipticity around 220 nm decreased slightly with increasing temperature. The CD spectrum of AAREP at 95 °C was stable for 24 h.

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Fig. 1. Comparison of AARE sequences. Sequence alignment was performed by a Genetyx-Mac program (Software Development Co., Ltd., Tokyo, Japan). The sequences have been aligned with dashes indicating gaps. Between two enzymes are marked with an asterisk (*). Putative active residues (Ser-491, Asp-572, and His-604) are marked +. Abbreviations: P. horikoshi, AARE from P. horikoshii; Pig, AARE from pig liver.

Fig. 2. SDS-PAGE (4–15% gradient gel) of the purified enzyme (lane 2). The following molecular mass standards were used (lane 1): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

2 K. Ishikawa and S. Tsunasawa, unpublished data.
Using DSC from 0 °C to 125 °C, we measured the heat capacity changes of AAREP. We observed two peaks of heat capacity changes of AAREP over 100 °C in the first scan (Fig. 6A) but no peak in the second scan. Precipitate was observed after the first scan. The temperature of the peaks was independent of the enzyme concentrations examined (0.1–2 mg/ml). This result indicates that the heat inactivation process of the enzyme is irreversible and accompanied by aggregation. The two peaks observed suggest that AAREP consists of two major domains as reported by Miyagi et al. (12).

These results indicate that incubating AAREP at 95 °C caused its structure to begin unfolding, but the major conformation of the enzyme remained stable from 0 °C to 100 °C.

**Effect of Heating**—After incubating at 95 °C, we measured the relative activity of AAREP at 85 °C to examine the effect of heating. Incubating at 95 °C appeared to increase the relative activity nearly 7-fold (Fig. 7). The enzyme did not lose its activity when incubated at 95 °C for 1 h.

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**TABLE I**

Substrate specificity of AAREP and HAAREP

| Substrate (10 mM) | Activity (µmol/min/mg enzyme) | Activity (µmol/min/mg enzyme) |
|------------------|--------------------------------|--------------------------------|
| Ac-Leu-pNA       | 105 ± 11                       | 750 ± 87                       | 45.3 |
| Leu-pNA          | <0.01                          | <0.01                          |      |
| Ac-Ala-pNA       | 6.9 ± 1.0                      | 51 ± 0.8                       | 457.3|
| Ala-pNA          | <0.01                          | <0.01                          |      |
| Ac-Tyr-pNA       | 1.9 ± 0.5                      | 15 ± 1.5                       |      |
| Ac-Met           | <0.01                          | <0.01                          |      |
| Ac-Met-Ala       | 344 ± 19                       | 5,120 ± 89                     | 47.1 |
| Ac-Ala-Ala       | 478 ± 60                       | 7,060 ± 507                    | 107  |
| Ac-Met-Ala-Ala-Ala-Ala | 11.7 ± 3.0                   | 77.0 ± 24                      |      |
| Ac-Ala-Ala-Ala   | 206 ± 46                       | 1,830 ± 277                    | 53   |
| Ac-Ala-Ala-Ala-Ala-Ala | 13.6 ± 7.8                   | 118 ± 20                       |      |
| f-Met            | <0.01                          | <0.01                          |      |
| f-Met-Ala-Ser    | 1,388 ± 188                    | 17,026 ± 295                   |      |
| f-Met-Ala        | 97.2 ± 10                      | 1,513 ± 182                    | 42.5 |
| f-Met-Leu-Gly    | 694 ± 78                       | 7,064 ± 550                    |      |
| f-Ala-Ala-Ala    | 402 ± 63                       | 5,383 ± 711                    |      |
| f-Met-Ala-Ala-Ala-Ala | 9.5 ± 1.6                     | 66.8 ± 18.7                    |      |
| f-Ala-Ala-Ala-Ala-Ala | 10.2 ± 2.6                     | 77.3 ± 14.0                    |      |

**TABLE II**

Kinetic parameters of AAREP and HAAREP

| Substrate     | AAREP          | HAAREP         |
|---------------|----------------|----------------|
|               | $K_m$ (mM)     | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM)     | $k_{cat}$ (s$^{-1}$) |
| Ac-Leu-pNA    | 11.0 ± 5.5     | 330 ± 31       | 12.9 ± 9.0     | 3,200 ± 230          |
| Ac-Ala-pNA    | 18.4 ± 4.0     | 173.3 ± 0.6    | 20.2 ± 5.7     | 144 ± 23             |
| Ac-Ala-Ala    | 7.6 ± 0.8      | 790 ± 155      | 8.6 ± 1.0      | 9,900 ± 611          |
| Ac-Ala-Ala-Ala-Ala | 13.0 ± 1.5     | 281 ± 44       | 15.2 ± 2.9     | 2,090 ± 230          |

**FIG. 3.** Effect of pH on the hydrolytic activity of AAREP (○) and HAAREP (●) on Ac-Leu-pNA. The hydrolytic activity was measured at 85 °C in 50 mM sodium acetate buffer (pH 4–5.6), 50 mM sodium phosphate buffer (pH 6–8), and 50 mM NaH$_2$PO$_4$-Na$_2$B$_4$O$_7$ buffer (pH 8–9), containing 0.6 M NaCl and 5% DMF. The assay was measured for 10 min.

**FIG. 4.** Effect of the temperature on the hydrolytic activity of AAREP (○) and HAAREP (●) on Ac-Leu-pNA. The hydrolytic activity was measured in 50 mM sodium acetate buffer (pH 5.4) containing 0.6 M NaCl and 5% DMF. The assay was measured for 10 min. Inset: Arrhenius plot.

**FIG. 5.** Far-UV CD spectra of AAREP at 25 °C (○) and 95 °C (●). The enzyme concentration was 0.23 mg/ml in 50 mM sodium acetate buffer (pH 5.4) containing 0.6 M NaCl and 5% DMF. The path length of the quartz cell was 5.0 mm. Three replications were completed to produce the data.
increased activity upon cooling (4–25 °C), suggesting that the activation was irreversible. This heat-activated enzyme (hereafter referred to as HAAREP) was also stable at 95 °C for 24 h.

From the light-scattering photometry, the molecular mass of HAAREP was determined to be 260 kDa, and the spatial size of the associated molecule was observed to be expanded in space compared with AAREP; its z-average radius of gyration ($R_G$) had increased from less than 100 to more than 400. These molecular mass and $R_G$ values were virtually constant for nearly 5 days (250–264 kDa and $R_G = 416–445$) at room temperature, without significant decomposition of the molecule or development of aggregation. The molecular mass value indicates that the number of monomers constituting the associated molecule averages a little more than 3 in this condition. However, the significant spatial expansion ($R_G = 416–445$) strongly suggests a conformational change over the monomeric structural unit as a result of heat treatment. By heating, the absorbance around 250–280 nm was increased by 10 ± 1.3%, and the intensity of the negative ellipticity of the CD around 220 nm was decreased slightly (Fig. 5). The changes in the absorbance and CD were parallel to the change in the activity of the enzyme. The NH$_2$-terminal sequence of HAAREP remained identical to that of AAREP. The rate of activation by heat treatment was independent of the enzyme concentrations examined (0.04–1.23 mg/ml). Therefore, it is deduced that the conformational change by heat treatment alters the molecular character of monomer and increases the activity. The NH$_2$-terminal section of about 500 residues (12) in the enzyme might be related to the conformational change by heat treatment. We are continuing to investigate these points.

In comparing the characteristics of the two enzymes, we found that HAAREP had a higher optimum pH, above 7.0 (Fig. 3) and a higher optimum temperature, 95 °C (Fig. 4). The relative specificity of HAAREP for substrates was similar to that of AAREP, although HAAREP showed a 7–14-fold increase in specific activity (Table I). The activation parameters of these enzymes were measured from 50–85 °C (Table III). The temperature dependence on the $K_m$ value of Ac-Leu-pNA for AAREP ($K_m$ values at 60, 75, 80, 85, and 90 °C were 0.689 ± 0.21, 3.60 ± 0.66, 3.85 ± 0.91, 11.0 ± 6.3, and 18.0 ± 5.6 mM, respectively) was similar to that for HAAREP ($K_m$ values at 60, 75, 80, 85, and 90 °C were 0.876 ± 0.24, 4.12 ± 0.51, 3.99 ± 0.66, 12.9 ± 9.0, and 19.7 ± 2.1 mM, respectively) (Table III). From the temperature dependence on the $k_{cat}$ value of Ac-Leu-pNA, the activation energy of HAAREP was found to be greater than that of AAREP (Fig. 4) and Table III). Both $\Delta G^\circ$, $\Delta H^\circ$, and $\Delta S^\circ$ values of the activation were increased by heat treatment. The shapes of the DSC curves of HAAREP (Fig. 6A) and AAREP (Fig. 6B) were slightly different from each other, but both enzymes seem to be stable below 100 °C. These results suggest that the conformational change in the enzyme by heat treatment has an orienting effect on the catalytic groups of the active site, making the enzyme more active at higher temperatures. It is speculated that HAAREP is a stable intermediate state between the native AAREP state and the heat-activated (unfolded) state of the enzyme. Although we have no information about the activity and structure of native AAREP in _P. horikoshii_ cells, HAAREP is thought to be the dominant state of the enzyme in _P. horikoshii_ because of the organism's high optimum growth temperature.

Until now, AAREP has been found only in eukaryotic cells and thought to be related to the initiation of protein synthesis (1,
21–23). The existence of this enzyme in the archaeon \textit{P. horikoshii} suggests that the initiation of protein synthesis in archaea is similar to that in eukaryotic cells. From the fact that a number of eukaryotic intracellular proteins are known to be N\textsuperscript{\alpha}-acylated (24, 25), it is speculated that many proteins of archaea are also N\textsuperscript{\alpha}-acylated. Furthermore, the existence of proteasomes in \textit{P. horikoshii} (26, 27) suggests that the action of the enzyme AAREP might be related to the ubiquitin/ATP-dependent system of protein degradation (28–31). Archaea also contains aminoacylase (26, 32), which might play an important role in the recycling of acylamino acids for protein synthesis with the help of AARE.

In eukaryotic cells, a strong degree of genetic similarity between AARE and aminoacylase was suggested by Jones \textit{et al.} (33). In \textit{P. horikoshii}, however, the gene for aminoacylase was found at another locus in the genome (27), and AAREP did not share homology or activity with aminoacylase.

AARE from mammals has been used to remove N\textsuperscript{\alpha}-acylamino acid residues from acylpeptides for protein sequencing at relatively low temperature (37 °C). AAREP may not be used to remove N\textsuperscript{\alpha}-acylamino acid residues of relatively long acylpeptides. However, AAREP is expected to be used for relatively short acylpeptides in sequence analysis at temperatures higher than 90 °C.

Studies about the crystal structure, thermostability, and hydrolytic mechanism of AAREP are in progress.

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