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Synthesis and Degradation of Acyl Peptide Using Enzyme from *Pseudomonas aeruginosa*

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The detailed properties of the enzyme from *Pseudomonas aeruginosa*, which catalyzes the *N*-acyl linkage between myristic acid and the *N*-terminal glycine residue of the octapeptide GNAAAARR-NH_2 (PKA) in aqueous solution without ATP and CoA, were studied. The substrate specificity for the acyl peptide in the synthetic reaction was examined, and it was found that at least eight amino acid residues are required for the reaction and that the *N*-terminal glycine residue is not absolutely essential for the reaction because the activity was detected using the octapeptide that has an *N*-terminal alanine. The activity was also strongly affected by the amino acid sequence because the activity was very weak in the reaction using GARASVLS-NH_2 (HIV-1p17^{gag}). The substrate specificity for fatty acids was also examined. In the reactions using lauric acid and decanoic acid, only slight activities were detected; however, those activities were very small compared with the activity in the reaction using myristic acid. In addition, the degradation of myristoyl PKA by the enzyme was detected, although there are only a few reports on demyristoylation. The optimum pH and temperature of the degradation reaction were consistent with those of the synthetic reaction. The degradation reaction was inhibited by divalent cations.

[**Key words:** *N*-myristoyl peptide, myristoylation, demyristoylation, *N*-acyl linkage, acylation, *Pseudomonas aeruginosa*]

Many myristoyl proteins involved in cellular regulation and/or signal transduction have been found. *N*-Myristoylation of protein is catalyzed by *N*-myristoyl transferase (NMT) using myristoyl-CoA as a substrate and ATP as an energy source, and myristic acid is attached via an amide bond to the *N*-terminal glycine residues of proteins (1). The modification is considered to be cotranslational, irreversible (2) and observed only in eukaryotic cells because no NMT and myristoyl protein have been yet identified in prokaryotes. NMTs of various eukaryotic origins have been found, and it was predicted that approximately 0.5% of all encoded proteins are apparent substrates of NMT from the analyses of complete eukaryote genomes (3). Moreover, NMT has attracted considerable attention as a target of antifungal and antiviral agents because NMT is essential for the viability of *Candida albicans* (4) and *Cryptococcus neoformans* (5) and the viral infectivity of human immunodeficiency virus type 1 (HIV-1) (6).

In a previous report, we found an enzyme from *Pseudomonas aeruginosa*, which catalyzes the *N*-myristoyl linkage between myristic acid and the octapeptide GNAAAARR-NH_2 (PKA) in the absence of ATP and CoA, and reported on the purification and characterization of such enzyme (7). This was the first report on *N*-myristoylation without CoA and ATP. The enzyme was purified about 1900-fold, and the molecular mass was determined to be approximately 60 kDa. The K_ms of the purified enzyme for myristic acid and the octapeptide (PKA) were 0.36 and 2.6 mM, respectively, and the affinity for the octapeptide was weak. It was difficult to specify the role of the enzyme *in vivo* from the information obtained in the previous paper, and more detailed analyses of the enzyme are required to clarify the role. As a possible application of the enzyme, it may be useful for the syntheses of various bioactive myristoyl peptides. Furthermore, ATP required for the reactions of many industrial enzymes is expensive, and understanding the mechanism of the ATP-independent reaction described here may provide valuable information for the improvement of ATP-dependent industrial enzymes.

Here, we examined the detailed properties of the purified enzyme from *P. aeruginosa*, particularly the substrate specificity in the synthetic reaction of acyl peptide and the properties of the degradation reaction of myristoyl PKA; several significant results for understanding the enzyme were obtained.

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Abbreviations: NMT, *N*-myristoyl transferase; PNM, *Pseudomonas N*-myristoylation enzyme.

MATERIALS AND METHODS

Materials The peptides GNAAAARR-NH_2 (PKA), GNAAAA-

NH_2 (PKA6), GNAA-NH_2 (PKA4), which are based on the *N*-terminal sequence of the type II catalytic subunit of the cAMP-dependent protein kinase, ANAAAARR-NH_2 (G→A PKA), in which the *N*-terminal glycine of PKA was replaced with alanine, GARASVLS-NH_2 (HIV-1p17^{gag}) (6) and GSSKSKPK-NH_2 (pp60^{SRC}) (8) were used for the enzyme reaction. These peptides were purchased from Greiner Bio-One, Tokyo. Myristic acid and other fatty acids were purchased from Wako Pure Chemical Industries, Osaka. DEAE–Cellulose (SERVA Electrophoresis, Heidelberg, Germany), Sephadex G-100 (GE Healthcare UK, Buckinghamshire, UK), CM Sephadex C-50 (GE Healthcare UK) and hydroxyapatite (Wako Pure Chemical Industries) were used for the enzyme purification. Other general laboratory reagents of analytical grade were purchased from Wako Pure Chemical Industries. Myristoyl PKA, which was used as a standard for the synthetic reaction and a substrate for the degradation reaction, was synthesized according to the method of Towler and Glaser (9).

Culture condition and purification of the enzyme Cell culture and enzyme purification were carried out according to the methods described previously (7). *P. aeruginosa* (strain OCU 169 provided by Tanabe Seiyaku, Osaka) was cultured for the enzyme production. For the purification of the enzyme, the cells were harvested from the culture broth by centrifugation (17,800×*g*, 20 min), suspended in potassium phosphate buffer (10 mM, pH 7.0), disrupted using an ultrasonic oscillator, and centrifuged. The supernatant was then applied to DEAE cellulose, Sephadex G-100, CM-Sephadex C-50 and hydroxyapatite column chromatographies. Here, Sephadex G-100 was used instead of Sephadex G-200, which was used in the previous study. Finally, the enzyme was purified 1800- to 2000-fold from the crude extract. The enzyme was stable for two months at 4°C.

Synthetic reaction of myristoyl peptide The synthesis of the myristoyl peptide was performed as described previously (7). The reaction was initiated by the addition of 25 µl of the enzyme solution (0.14 or 0.075 unit) to 85 µl of the reaction mixture containing 33 mM borate buffer (pH 9.0), 7.1 mM myristic acid, and 3.5 mM octapeptide. The phosphate buffer (10 mM, pH 7.0) was used instead of the enzyme solution for the control experiments. The mixture was incubated at 40°C for 20 min. One unit of activity was defined as the formation of 1 nmol *N*-myristoyl octapeptide per min.

To examine the substrate specificity in the synthetic reaction of myristoyl peptide, peptides (PKA, PKA6, PKA4, G→A PKA, HIV-1p17^{gag} and pp60^{SRC}) and fatty acids (lauric acid, decanoic acid, palmitic acid, and stearic acid) were used as a substrate. After the reaction, HPLC analyses of the reaction mixtures were performed (7). The reaction solutions (supernatants after centrifugation) were also analyzed using MASS spectrometry; 0.5 µl of sample diluted 10 times with distilled water was analyzed using Shimadzu Kratos PC Axima CFRplus, using α-cyano-4-hydroxy cinnamic acid as a matrix. After the sample was dried on a stainless plate, it was ionized, and analyses of single MASS (Reflectron MALDI-TOF MASS) and/or MASS/MASS (PSD MALDI-TOF MASS) were performed.

Degradation reaction of myristoyl PKA The degradation of myristoyl PKA was examined as described below. The reaction was initiated by the addition of 25 µl of the enzyme solution (1.4×10^{-4} or 0.75×10^{-4} unit) to 85 µl of reaction mixture containing 40 mM borate buffer (pH 9.0) and 0.021 mM myristoyl PKA. In the case of the time-dependent experiment, the mixture was incubated at 40°C for 0–60 min. The reaction was terminated by the addition of 10 µl of 100% trichloroacetic acid and 110 µl of acetonotrile. HPLC and MASS analyses were performed using the same method used in the analysis of the synthetic reaction. To determine the suitable degradation temperature, the reaction mixtures were incubated at 10°C, 20°C, 40°C, 60°C and 80°C for 10 min. To determine the effect of pH on the degradation, acetate buffer (pH 4, 5, and 6), Tris–HCl buffer (pH 7) and borate buffer (pH 8, 9 and 10) were used. The reaction was performed at 40°C for 10 min in the solutions (110 µl) containing 0.016 mM myristoyl PKA and 31 mM of each buffer. To determine the effects of chemicals and ions on the degradation reaction, the reaction was performed at 40°C for 10 min in the reaction mixture containing an additional chemical or ion. After the reaction, HPLC analyses were performed using the method used in the analysis of the synthetic reaction.

For the measurement of the time course of the degradation, the reaction was performed at 40°C for 2–10 min in the solutions containing the enzyme (0.75×10^{-4} unit), 31 mM borate buffer (pH 9) and 0.016 or 0.066 mM myristoyl PKA. After the reaction, HPLC analyses were performed.

RESULTS

Substrate specificity of CoA- and ATP-independent *N*-myristoylation enzyme We previously found a new enzyme from *P. aeruginosa*, which catalyzes the *N*-myristoyl linkage between myristic acid and PKA in the absence of ATP and CoA as described in the introduction (7). Here, we call the enzyme PNM (*Pseudomonas N*-myristoylation enzyme) for convenience. PNM could use myristoyl-CoA instead of myristic acid as a substrate for myristoyl PKA synthesis (7). However, details on the substrate specificity of PNM are yet unclear. Therefore, we examined the effect of the amino acid sequence of the peptide and length of the saturated carbon chain of the fatty acid on the enzyme reaction. After the enzyme reactions using various substrates, the reaction products were analyzed by HPLC. In several substrates, new peaks appeared after the reaction as shown in Table 1. In addition, those products were analyzed by MASS spectrometry to confirm whether the acyl peptide appeared (Table 1). Results of representative MASS analyses are shown in Fig. 1.

At first, we examined the effect of peptide length using PKA, PKA6 and PKA4. In the HPLC analyses a new peak after the reaction using myristic acid and PKA was detected at the retention time of 9.0 min; however, new peaks were not detected after the reaction using PKA6 and PKA4 instead of PKA (Table 1). In the MASS analyses the molecular ion peak of myristoyl PKA (Fig. 1A) was detected at *m/z* 995.63; however, the peaks of myristoyl PKA6 (Fig. 1B) and myristoyl PKA4 were not detected. In all the control experiments without PNM, no peaks of acyl peptides were detected in the MASS analyses (data not shown). These results suggest that at least eight amino acid residues are required for the reaction by PNM.

Next, we examined the effect of the amino acid sequence of the octapeptide using pp60^{SRC} and HIV-1p17^{gag}, which have *N*-terminal glycine. It is known that these peptides can be used as a substrate by NMT (6, 8). In the HPLC analyses of the reaction products using pp60^{SRC} and HIV-1p17^{gag}, the new peaks that appeared after those reactions were detected at the retention times of 6.5 and 11.2 min, respectively. However, the peak area using HIV-1p17^{gag} was very small compared with those using PKA and pp60^{SRC} (Table 1). In the MASS analyses, the molecular ion peaks of myristoyl pp60^{SRC} (Fig. 1C) and myristoyl HIV-1p17^{gag} were detected; however, the peak strength of myristoyl HIV-1p17^{gag} was