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Long-Range Effect of Mutation of Calcium Binding Asparates on the Catalytic Activity of Alkaline Protease from *Pseudomonas aeruginosa*[1]

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Apart from a catalytic domain, the alkaline protease of *Pseudomonas aeruginosa* has a novel parallel β-helix domain stabilized through Ca^{2+} binding. In order to clarify the importance of the β-helix structure in maturation of the enzyme, aspartic residue D-356 or D-365 in the Ca^{2+} binding sequence motif was replaced with L-alanine, and the catalytic activity of each mutant was assayed. These mutants did not show any proteolytic activity, although the composition of their polypeptide chains was the same as that of the wild type except for the mutated alanine residue. These results suggest that D-356 and D-365 are important in control of the β-helix folding induced by Ca^{2+} binding and that incomplete β-helix folding due to the lack of their side-chains affects the maturation of the enzyme in the long-range order.

Key words: alkaline protease, β-helix structure, Ca-binding sequence motif, *Pseudomonas aeruginosa*, site-directed mutagenesis.

Pseudomonas aeruginosa is an opportunistic pathogen which can cause a fatal infection in a vulnerable host. The roles of several extracellular metabolites have been established or implicated in the pathogenicity of this organism (*1*). Among the metabolites, alkaline protease (AP) has been studied extensively in relation with the virulence of the organism, the pathogenicity being now explained as aggressin activity (*2*).

P. aeruginosa can secrete AP through a one-step pathway system (*3*), similarly to as seen for the proteases of *Erwinia chrysanthemi* and α-hemolysin of *Escherichia coli*. AP can thus be produced extracellularly on growth in semi-synthetic or synthetic medium containing glucose, where the Ca^{2+} ions are essential components (*4*). An isotope experiment involving resting cells of *P. aeruginosa* indicated that the Ca^{2+} ions added to a medium containing only a carbon source were incorporated into the enzyme molecule (*5, 6*). The tightly bound Ca^{2+} ions could not be removed by treatment with EDTA (*7*).

AP is classified as a Zn-metalloprotease belonging to the serralysin family, having an extended Zn binding sequence motif, HExxHxxGxxH (x, an arbitrary amino acid residue) (*8, 9*). Recent studies on AP involving X-ray analysis (*10, 11*) have indicated that AP has a novel "parallel β-helix" structure in which 21 successive β-strands are wound in a right-handed spiral, and in which eight Ca^{2+} ions are bound within the turns between strands by a repeated GGxGxDxUx sequence motif (U, a large and hydrophobic amino acid residue). The sequence motif can also be seen in proteases or α-hemolysin (*3*) which are secreted through a one-step pathway system, as mentioned above.

Of the Ca^{2+} ions in AP, the three Ca^{2+} ions (Ca3-Ca5) in the central β-helix are buried internally between loops connecting consecutive β-strands and bound so strongly that they can not be removed from the protein with EDTA. Therefore, these Ca^{2+} ions seem to be essential determinants of the protein folding in the β-helix structure. The aspartic residues in the Ca^{2+}-binding sequence motif, especially D-356, D-365, and D-374, may play an important role in the incorporation of these Ca^{2+} ions into the enzyme, because the side-chain of each of these residues bridges loops in the central β-helix by ligating two Ca^{2+} ions. D-356 participates in the binding of Ca3 and Ca5, D-365 in the binding of Ca4 and Ca6, and D-374 in the binding of Ca5 and Ca7. In order to clarify the importance of the β-helix in maturation of the enzyme, mutants were prepared by replacing D-356 and D-365 with L-alanine, respectively, and then the catalytic activity of the mutants was assayed.

The various plasmids used were as follows. pAPE1 was constructed by inserting an *Eco*RI-*Pst*I fragment (2.0 kb) derived from pAPS93 into pUC18. pAPS93 was obtained by colony hybridization using an *Eco*RV fragment (0.9 kb) from pAPDS2 (*12*) as a probe and contains a 7 kb fragment including the entire *apr*A (structural gene for AP) sequence, which was generated by *Sau*3AI partial digestion of *P. aeruginosa* IFO3455 genomic DNA. pAPDS2 was origi-

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nally constructed from pAP101, which was selected by expression screening using anti-AP antibodies and contained the 5′-65 bp truncated *apr*A open reading frame, by deleting the 3′ unrelated sequence using exonuclease III to the 0.6 kb downstream from the *Pst*I site (*12*). Figure 1 shows the plasmids used in this experiment, which contain the coding region for *apr*A.

Site-directed mutagenesis was carried out by the protocol described by Kunkel (*13*), using a reaction kit (Mutan-K; Takara Shuzo, Kyoto). Various restriction enzymes and plasmid vector pUC18 were also supplied by Takara Shuzo. A *Kpn*I-*Pst*I fragment (1.3 kb) from pAPE1 including the codons to be mutagenized was inserted into M13mp19 RF DNA. Single-stranded DNA was prepared and subjected to site-directed mutagenesis with the following oligonucleotides: 5′-CCGGCAACGCCATCCTCTA-3′ (D356→A) and 5′-TCGGCGCGGCCCAGTTGTG-3′ (D365→A). The oligomers were synthesized using Pharmacia LKB Gene Assembler Special. RF DNA was prepared from the mutagenized phage clones, and the mutated *Kpn*I-*Pst*I fragment was ligated into expression vector pAPE1, which lacked the corresponding *Kpn*I-*Pst*I fragment. The mutations were finally confirmed by DNA sequencing using a M13 universal primer (5′-fluorescein labeled) by the dideoxy chain termination method (*14*), both *Ava*I-*Pst*I (0.8 kb) and *Kpn*I-*Ava*I (0.5 kb) fragments of mutated pAPE1 being sequenced with a Shimadzu DNA sequencer (DSQ-1) using an Auto Read™ Sequencing kit (Pharmacia Biotech., Tokyo).

The *E. coli* transformant with pAPE1 which contains the *apr*A gene produced AP in neither the cells nor the culture broth. However, *E. coli* transformed with plasmid pAPDS2 can produce AP intracellularly, as was described in the previous paper (*12*). The discrepancy will be discussed later. So, a *Sph*I-*Pvu*II fragment (1.0 kb) of mutant pAPE1 was inserted into pAPDS2, which lacked the corresponding *Sph*I-*Pvu*II fragment. The thus obtained mutants (pAPDA356 and pAPDA365) were used for the following experiment.

E. coli (JM109) cells were transformed with the above vectors, and then the transformants were cultured in LB medium (5 ml) containing ampicillin (50 μg/ml) with shaking at 37°C. Isopropyl-β-D-thio-galactopyranoside (1 mM) was added at the mid-log phase, and then cultivation was continued for 16 h. Cells were collected, washed, and then resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$. These suspensions were sonicated with four 30-s pulses and then centrifuged (10,000 rpm, 15 min). The supernatant was used for the following experiment. The precipitate was solubilized with 1 ml of 8 M urea and then centrifuged as above. The supernatant was dialyzed against 10 mM Tris-buffer (pH 8.0), which was used as the precipitate fraction.

The proteolytic activity of both fractions was determined using casein as follows. A 1 ml aliquot of a 5-fold diluted solution of each fraction, with 10 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$, was mixed with 1 ml of a 2% casein solution (Hammarsten, adjusted to pH 7.4), which was kept for 30 min at 40°C. After the reaction, 2 ml of a 10% trichloroacetic acid solution was added to the reaction mixture, followed by centrifugation. The absorbance of the supernatant was read at 280 nm. The results are summarized in Table I, which indicates that the proteolytic activity was detected in both fractions of sonicated *E. coli* cells transformed with plasmid pAPDS2. However, the previous study (*12*) involving the same *E. coli* (strain HB101) transformant indicated that the proteolytic activity was only detected in the precipitate fraction, in which AP was probably accumulated as an insoluble aggregate. This discrepancy may be ascribed to the difference in the *E. coli* strains used for transformation or the time of sonication. Anyway, it can be considered that the insoluble aggregate became partly soluble with the sonication treatment. On the other hand, none of the proteolytic activity was detected in the fractions of *E. coli* cells transformed with the mutant genes.

Fractions of sonicated bacteria were analyzed by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis. To confirm the presence of the AP protein, Western immunoblotting was performed according to the procedure of Burnette (*15*). Anti-AP antibodies were prepared according to the method described previously (*12*). Peroxidase-linked anti-rabbit Ig, secondary antibodies, was supplied by Takara Shuzo. The results of

TABLE I. **Proteinase activities of bacterial sonicated fractions.**

Bacterial strain	Proteinase activity[a] of:	
	Supernatant	Precipitate
E. coli (pAPE1)	0.010	0.006
E. coli (pAPDS2)	0.076	0.145
E. coli (pAPDA356)	0.009	0.005
E. coli (pAPDA365)	0.006	0.008

[a]The optical density at 280 nm.

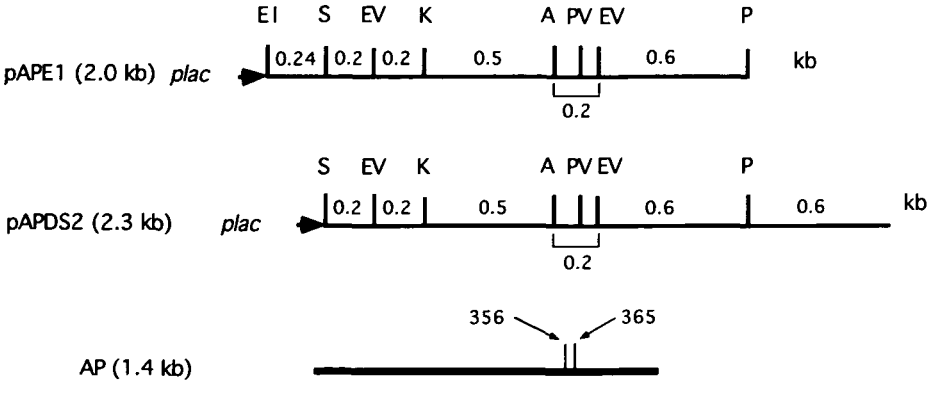

Fig. 1. **Restriction endonuclease map of plasmids pAPE1 and pAPDS2.** The molecular sizes of the fragments inserted into pUC 18 are given in parentheses. The thick line indicates the gene corresponding to AP. The arrows for the AP gene indicate the sites corresponding to D-356 and D-365. A, *Ava*I; EI, *Eco*RI; EV, *Eco*RV; S, *Sph*I; K, *Kpn*I; P, *Pst*I; PV, *Pvu*II.

Western blotting are shown in Fig. 2. It indicated that all the products of the wild and mutant genes are the same, being somewhat larger proteins (*ca.* 51 kDa) than AP. A similar case has been observed for the product of an *E. coli* transformant with pAP101 (*12*). The above experiment was also performed using *E. coli* cells harboring plasmid pAPE1, but a band (49 kDa) corresponding to AP was never detected on Western blotting (data not shown).

Guzzo *et al.* (*16*) have shown that *E. coli* cells carrying a plasmid constructed with only the *apr*A gene could not accumulate AP inside the cells, because the cell-bound protease was very unstable. By means of pulse-chase experiments, they showed that the protease was synthesized but was degraded very rapidly within the cells. This may be true in the present study for *E. coli* cells containing pAPE1, the bacteria showing negligible protease activity inside the cells, as can be seen in Table I. In contrast, *E. coli* cells containing plasmid pAPDS2 can accumulate AP inside the cells, possibly as an insoluble aggregate which prohibits auto-degradation. The primary sequence data for AP (*17*) indicate that AP produced by the pAPDS2 plasmid lacks the N-terminal 14 amino acid residues. An X-ray study of AP (*10, 11*) indicated that the N-terminal 17 residues are not involved in the globular domain which constitutes the active site. These findings may lead to the consideration that pAPDS2 must have been expressed as a fusion protein of the N-terminal truncated AP linked with the N-terminal 21 amino acid residues of lacZ derived from vector pUC18. The finding in Fig. 2 that the polypeptide chain length of the product derived from pAPDS2 gene is larger than that of AP may be explained by the increase of 7 amino acid residues in the fusion protein.

The present mutagenesis study indicated that either D-356 or D-365 is indispensable for the proteolytic activity of AP. In the structure of AP, these residues are located far from the active site, with distances of 39.48 and 39.06 Å between the active site zinc ion and the Ca^{2+} ions, Ca3 and Ca4, bound nearer to the zinc, respectively, and are not in direct contact with the active site. These findings imply that the absence of carboxyl side-chains at these positions of AP may result in imperfect β-helix folding, which leads to an inactive conformation of the active site in the long-range order. The crystal structure of AP (*10, 11*) allows us to speculate how the polypeptide chains of these mutants fold into such an inactive conformation, although the details of their folding are unclear. As is the case with AP, Ca^{2+} ions internally bound to the protein should be incorporated into its inside before completion of its folding, and may induce the proper folding of the enzyme. If the aspartic residue in the Ca^{2+}-binding motif of AP plays an important role in the incorporation of the Ca^{2+} ions, the absence of this residue is assumed to result in imperfect folding of the protein. Thus a mutant having alanine substituted for D-356 or D-365 is supposed to lose the abilities of incorporating the Ca^{2+} ions at the appropriate positions and of folding of the central β-helix into the proper conformation. Since the central β-helix is a quite rigid and regular structure, it is presumed that the central β-helix could control the irregular folding of other regions of the C-terminal β-helix domain. Consequently, the C-terminal domains of the mutants may have distorted β-helices or denatured conformations due to the lack of some Ca^{2+} ions. It is unclear why the imperfect conformation of the C-terminal domain causes the inactive conformation of the N-terminal catalytic domain. However, the N-terminal α-helix strongly in contact with the C-terminal domain may have an effect on the conformation of the catalytic domain. This helix extends far from the N-terminal catalytic domain to become in strong contact with the C-terminal β-helix domain. When the C-terminal domain has a denatured or imperfect conformation, the N-terminal α-helix may become quite flexible due to the loose contact with the C-terminal domain. The flexibility of the N-terminal α-helix, along with the imperfect folding of the C-terminal domain, could induce an inactive conformation of the catalytic domain. Therefore, a mutant having alanine substituted for D-356 or D-365 may have both imperfect C-terminal folding and an inactive N-terminal conformation, which could result in an inactive enzyme structure. As described above, D-356 and D-365 are important in control of the β-helix folding induced by Ca^{2+} binding, and the incomplete β-helix folding due to the lack of their side-chains affects the maturation of the enzyme in the long-range order.

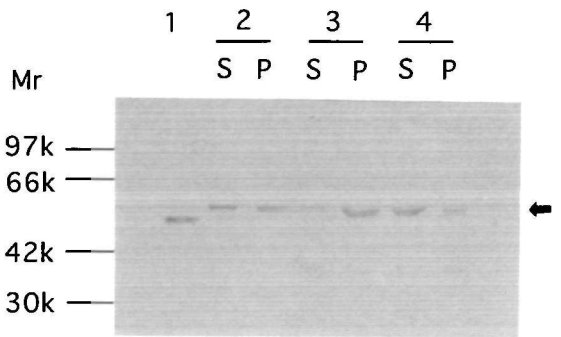

Fig. 2. **Immunodetection of alkaline protease in cellular extracts of *E. coli* cells carrying various plasmids.** S, supernatant of sonicated cells; P, precipitate of sonicated cells. Lanes: 1, purified alkaline protease; 2, *E. coli* (pAPDS2); 3, *E. coli* (pAPDA356); 4, *E. coli* (pAPDA365).

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