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Requirement for the COOH-Terminal Pro-Sequence in the Translocation of Aqualysin I across the Cytoplasmic Membrane in *Escherichia coli*

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Aqualysin I from *Thermus aquaticus* YT-1 is an extracellular subtilisin-type serine protease. The protease is synthesized as a distinct precursor composed of four functional domains: an N-terminal signal sequence, an N-terminal pro-sequence, a protease domain, and a C-terminal pro-sequence. The N-terminal pro-sequence is essential for the production of active aqualysin I while the C-terminal pro-sequence is required for extracellular secretion of aqualysin I. In an *E. coli* expression system, the function of C-terminal pro-sequence in the translocation of aqualysin I across the cytoplasmic membrane was investigated. More than 60–70% of the total activity was detected in the cytoplasmic fraction in the deletion mutations of the C-terminal pro-sequence while less than 30% was found in this fraction in wild type. In addition, *in vitro* processing of aqualysin I precursors with these mutations to a mature form promptly occurred and the folding into active aqualysin I was rapid. These results suggest that the C-terminal pro-sequence, probably in conjunction with the signal sequence, facilitates the translocation of the precursor across the cytoplasmic membrane by preventing the precursor from taking on an active conformation.

Key Words: **aqualysin I; serine protease; C-terminal pro-sequence; protein secretion; protein translocation; intramolecular chaperone.**

There are a large number of proteins synthesized in the form of transient precursors with an N-terminal and/or a C-terminal pro-sequence, often called intramolecular chaperones (1, 2). The precursors usually have a signal peptide. These pro-sequences are known to be required for the proper function of proteins to which they are attached, and processed after having performed their roles. The N-terminal pro-sequences are indispensable for proper folding of many proteins (2). On the other hand, in the cases of aqualysin I from *Thermus aquaticus* YT-1 (3, 4), IgA protease from *Neisseria gonorrhoeae* (5) and serine protease SSP from *Serratia marcescens* (6), the C-terminal pro-sequences are required for extracellular secretion of the proteases. The C-terminal pro-sequences of IgA (5) and SSP (6) proteases are particularly required for translocation of the proteases across the outer membrane.

T. aquaticus YT-1, an extremely thermophilic Gram-negative bacterium, secretes aqualysin I (a subtilisin-type alkaline serine protease) to the extracellular space (7–9). Aqualysin I is produced from pre-pro(N)-aqualysin I-pro(C) form (10) consisting of the signal peptide of 14 residues presumably required for protein translocation across the inner bacterial membrane, the N-terminal pro-sequence of 113 residues essential for protein folding (11), mature aqualysin I of 281 residues, and the C-terminal pro-sequence of 105 residues required for protease secretion (3, 4). The processing of the precursor occurs in the NH_2 to the COOH terminus order (10). The signal peptide is cleaved off by a signal peptidase. The N-terminal pro-sequence is thought to be removed through a *cis* (intramolecular) autoprocessing mechanism while the C-terminal pro-sequence is autoprocessed via a *trans* (intermolecular) mechanism after having performed their functions (4, 12). The precursor with the N-terminal pro-sequence is not detected because the pro-sequence is rapidly processed from the mature polypeptide, probably due to a very small *K*m for the processing (10, 12). In contrast, the C-terminal pro-sequence of aqualysin I precursor is detected in the form of a precursor consisting of a mature polypeptide and a C-terminal pro-sequence in *T. aquaticus* YT-I and *T. thermophilus* (4), or *E. coli* (10).

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TABLE I

Oligonucleotides Used for Site-Directed Mutagenesis

Mutation	Oligonucleotide*
C5A	5′-CACAGCCCCCGCTACCAGATGT-3′
C5S	5′-ACAGCCCCCTCTACCAGATGT-3′
C8A	5′-CTGTAC**CAGCGC**TAGCTACTAC-3′
C8S	5′-CTGTAC**CAGCTC**TAGCTACTACA-3′
C5A/C8A	5′-CACAGCCCCCGCTAC**CAGCGC**TAGCTACTAC-3′
C5S/C8S	5′-ACAGCCCCCTCTAC**CAGCTC**TAGCTACTAC-3′
Δ CM20	5′-TCCGGTCCTGGTGAC#TACAGCCCTGCAGGT-3′
Δ C30	5′-ACCTCGGAGGAAAGT#TAGGCGAAGGAGTTC-3′

* Single underlines show substituted bases from TG or G to GC or C, respectively. # and double underlines indicate the truncated positions and termination codon of *aquI* gene, respectively. Sequences in bold mark the changed *Pvu*II site (CAGCTG).

In the *E. coli* expression system for the wild-type aqualysin I, the 38-kDa pro-aqualysin I with the C-terminal pro-sequence accumulates in the membrane fraction and is detected in the outer membrane after translocation across the cytoplasmic membrane (10). In order to investigate the effect of the C-terminal pro-sequence on translocation of aqualysin I across the cytoplasmic membrane in an *E. coli* expression system, we examined the localization of aqualysin I precursors with mutations in the C-terminal pro-sequence. In addition, *in vitro* processing from precursors with the mutations to a mature protein and proteolytic activation of aqualysin I were investigated. We report here that the C-terminal pro-sequence affects the translocation of aqualysin I across the cytoplasmic membrane despite the presence of its signal peptide.

MATERIALS AND METHODS

Bacterial strain, growth conditions, and preparation of assay samples. *E. coli* MV1184 cells (10) were used as a host for expression of *aquI* and its mutated genes, DNA preparation and phage M13 propagation. *E. coli* MV1184 cells carrying various recombinant plasmids were inoculated and cultured at 37°C overnight in a modified L-broth medium (1% tryptone, 0.2% yeast extract, 0.5% NaCl) containing 1% glucose and 100 μg/ml of ampicillin. Precultured cells were inoculated (about 5%) into the same medium and cultured at 37°C. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the culture medium at a final concentration of 0.05 mM at an OD_{660} of 0.8 and expression of aqualysin I was induced for 2 h. The induced cells were harvested, suspended in 50 mM Hepes buffer (pH 7.5) containing 10 mM $CaCl_2$, and disrupted by sonication. The sonicated cells were heat-treated at 65°C up to 12 h for processing and activation of aqualysin I precursor and then, after removal of denatured proteins by centrifugation for 5 min at 10,000*g*, each supernatant was used for measurement of proteolytic activity, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

Recombinant DNA techniques and plasmid construction. Standard molecular biology methods were as described in Sambrook *et al.* (13). The oligonucleotides (Table I) for site-directed mutagenesis were synthesized by Applied Biosystem 381A DNA Synthesizer and monomers for the synthesis were purchased from Applied Biosystem, Inc. To produce a single-stranded DNA template for mutagenesis, the 0.68-kb *Xba*I-*Hin*dIII fragment of pAQN (11), an *E. coli* expression vector for the wild-type aqualysin I gene, was introduced into the M13mp19. Site-directed mutagenesis was accomplished using the MUTA-GENE *in vitro* mutagenesis kit (Bio-Rad), and the mutations were confirmed by restriction enzyme analysis and dideoxy chain termination sequencing. The plasmid pAQN was used to construct other pAQN derivatives (Fig. 1) by using endogenous restriction sites. In order to make pAQNΔ C72, the 0.68-kb *Xba*I-*Hin*dIII fragment of plasmid pAQN was inserted into the 3.1-kb *Xba*I-*Hin*dIII fragment of pUC119, digested with *Pst*I and *Sty*I, filled in with Klenow and self-ligated. The shortened 0.5-kb *Xba*I-*Hin*dIII fragment of pUC119 was used for replacement of the corresponding 0.68-kb *Xba*I-*Hin*dIII fragment of pAQN to yield pAQNΔ C72. The construction of other plasmids, pAQNΔ C5 (3), Δ C10 (3), Δ CN10 (4), Δ C20 (4), Δ CM30 (4), Δ C39 (4) and Δ C105 (14), was described previously.

Enzyme activity measurement. The proteolytic activity of aqualysin I was assayed as described previously (14). β-Lactamase activity was assayed at 30°C using nitrocefin dissolved in 50 mM Tris–HCl buffer (pH 7.5) at a final concentration of 60 μM as a substrate. Initial reaction rates were determined by measuring absorbency at 390 nm. One unit of the activity was defined as the amount of enzyme hydrolyzing one μmol of the substrate per min.

SDS–PAGE and Western blotting. Enzyme samples were treated with phenylmethylsulfonyl fluoride at a final concentration of 5 mM at room temperature for 30 min to prevent activation and autocatalysis of aqualysin I before addition of sample buffer, and then fractionated by SDS–PAGE on a 12% acrylamide gel according to the method of Laemmli (15). Immunoblotting analysis of aqualysin I and its precursors was carried out using an antibody against aqualysin I as described previously (10).

Fractionation into cytoplasmic, periplasmic, and membrane portions of E. coli cells. Subcellular fractionation of *E. coli* cells was carried out by modifying the method of Cornelis *et al.* (16). *E. coli* cells induced at an OD_{660} of 0.8 with 0.05 mM IPTG for 2 h at 30°C were harvested and washed twice with 20 mM Tris–HCl buffer (pH 7.5). The washed cells were suspended in the same buffer containing 25% (w/v) sucrose and 1 mM EDTA. The suspension was incubated with slow shaking for 10 min at room temperature. After harvesting, the cells were resuspended in cold distilled water and then incubated for 10 min at 4°C with occasional vigorous shaking. After centrifugation at 12,000*g* for 5 min, the supernatant was used as a periplasmic fraction, and the pellets were washed and suspended in 50 mM Hepes (pH 7.5) buffer containing 10 mM $CaCl_2$. After disruption of the pellets by sonication, the debris was removed by centrifugation at 3000*g* for 5 min and the supernatant was divided into the cytoplasmic and the membrane fractions by ultracentrifugation at 100,000*g* for 1 h at 4°C. The membrane fraction was washed twice with the same buffer. Each fraction was heat-treated at 65°C for 3 h for activation of aqualysin I and, following removal of denatured proteins by centrifugation for 5 min at 10,000*g*, each supernatant was used for measurement of aqualysin I activity. β-Lactamase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activities (data not shown) were measured as marker enzymes for the periplasmic and cytoplasmic fractions, respectively.

RESULTS AND DISCUSSION

Effects of the C-terminal pro-sequence mutations on the translocation of aqualysin I across the cytoplasmic membrane. The C-terminal pro-sequence of aqualysin I is required for its extracellular secretion by *Thermus thermophilus* (3, 4). In the *E. coli* expression system, aqualysin I is located in the outer membrane after translocation across the cytoplasmic membrane (10). In order to examine the effect of its C-terminal pro-

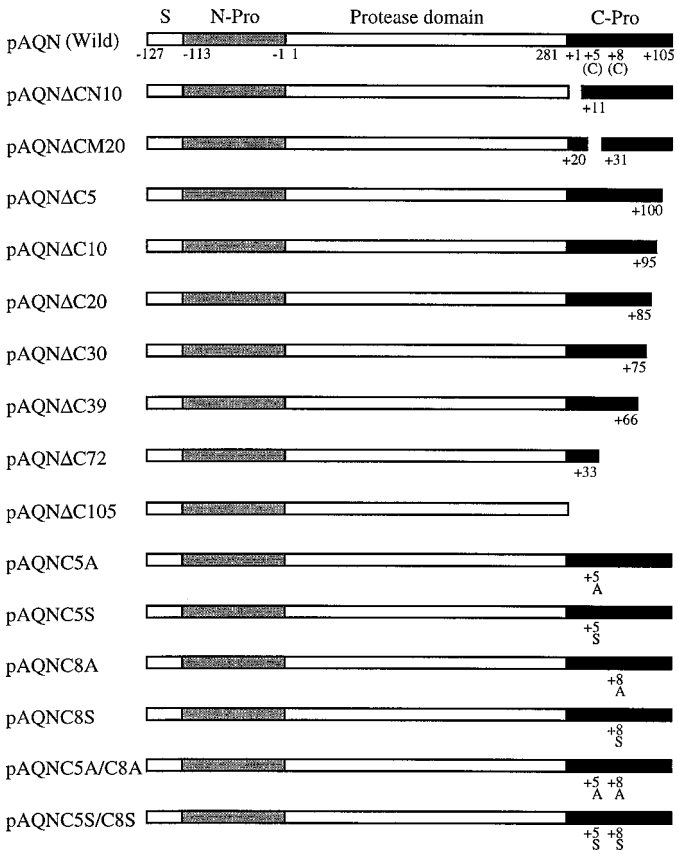

FIG. 1. Mutations in the C-terminal pro-sequence of aqualysin I precursor. pAQNΔ CN10 ~ Δ C105 are plasmids for aqualysin I precursors with deletions in the C-terminal pro-sequence, and pAQNC5A ~ C5S/C8S are substitution mutations of two cysteine residues at positions +5 and/or +8 with alanine or serine residues, respectively. WT, wild type; S, signal peptide; N-pro, N-terminal pro-sequence; C-pro, C-terminal pro-sequence. The numbers refer to positions of amino acid residues. The plasmid pAQN (11) has pUC *ori* and ampicillin-resistance gene. The *aquI* gene on the plasmid is under the control of *tac* promoter.

sequence on the translocation of aqualysin I across the cytoplasmic membrane in *E. coli* cells, the cells harboring plasmids for wild-type and mutant aqualysin I genes (Fig. 1) were cultured with IPTG to induce aqualysin I expression and fractionated into periplasmic, cytoplasmic and membrane fractions. After activation of aqualysin I by heat treatment, the proteolytic activity of each fraction was measured (Table II). In the cases of the wild-type and substitution mutations, approximately 70–72% of the total activity was found in the membrane fraction (Table II) and aqualysin I was confirmed to be in the outer membrane by sucrose density gradient centrifugation assay (data not shown). In contrast, in the case of a complete C-terminal pro-sequence deletion mutation (Δ C105), more than 70% of the total activity was detected in the cytoplasmic fraction. More than 60% of the total activity of partial deletion mutations, with the exception of Δ CN10 (see below), was in the cytoplasm fraction. This is similar to the case of a complete C-terminal pro-sequence deletion mutation, indicating that the C-terminal pro-sequence of aqualysin I is required for the translocation of the protease across the cytoplasmic membrane in *E. coli* cells even though aqualysin I contains the signal peptide. Deletion of any part of the C-terminal pro-sequence showed similar results, indicating that the overall structure of the pro-sequence is important for function. In all cases, little proteolytic activity was detected in the periplasmic fraction. The specific activities of mature proteases produced from wild type and mutants were the same (data not shown).

In vitro processing and proteolytic activation of aqualysin I precursors with the C-terminal pro-sequence mutations. In *T. aquaticus* YT-I and *T. thermophilus* expression systems for wild-type aqualysin I, a pro-aqualysin I with only C-terminal pro-sequence was initially detected in the culture medium and then the pro-sequence was processed in the medium (4). The pro-aqualysin I with the C-terminal pro-sequence was also detected in the membrane fraction when it was expressed in *E. coli* (10), and it was processed to a mature form by heat treatment, indicating that the C-terminal pro-sequence was more stable compared to the N-terminal pro-sequence, which was rapidly processed without heat treatment.

In order to investigate the effect of C-terminal pro-sequence mutations on the maturation rate of aqualysin I, the conversion of a 38-kDa precursor with the

TABLE II

Aqualysin I Localization in *E. coli* Cells

Enzymes	Mutations	Distribution of proteolytic activity (%)		
		Cytoplasm	Periplasm	Membrane*
Aqualysin I (β-lactamase)	pAQN (WT)	27 (8)	1 (92)	72 (ND)
	C5A/C8A	28 (10)	3 (90)	70 (ND)
	C5S/C8S	27 (9)	2 (91)	71 (ND)
	Δ CN10	16 (6)	1 (94)	83 (ND)
	Δ C5	60 (10)	3 (90)	37 (ND)
	Δ C10	63 (11)	2 (89)	35 (ND)
	Δ CM20	61 (8)	1 (92)	38 (ND)
	Δ C20	62 (10)	2 (90)	36 (ND)
	Δ C39	60 (9)	1 (91)	39 (ND)
	Δ C105	73 (10)	3 (90)	24 (ND)

Note. E. coli cells harboring wild-type and mutant plasmids were cultured for 2 h at 37°C in modified L-broth medium with 0.05 mM IPTG for aqualysin I induction, fractionated, and heat-treated at 65°C for 3 h for activation of the aqualysin I. Aqualysin I activity of each fraction was measured and expressed as a percentage of total activity. β-Lactamase and GAPDH (data not shown) activities as periplasmic and cytoplasmic markers were measured (%) and it was confirmed that the fractionation was successful. ND indicates that activity was below 1%. * Aqualysin I was confirmed to localize to the outer membrane by sucrose density gradient centrifugation (data not shown).

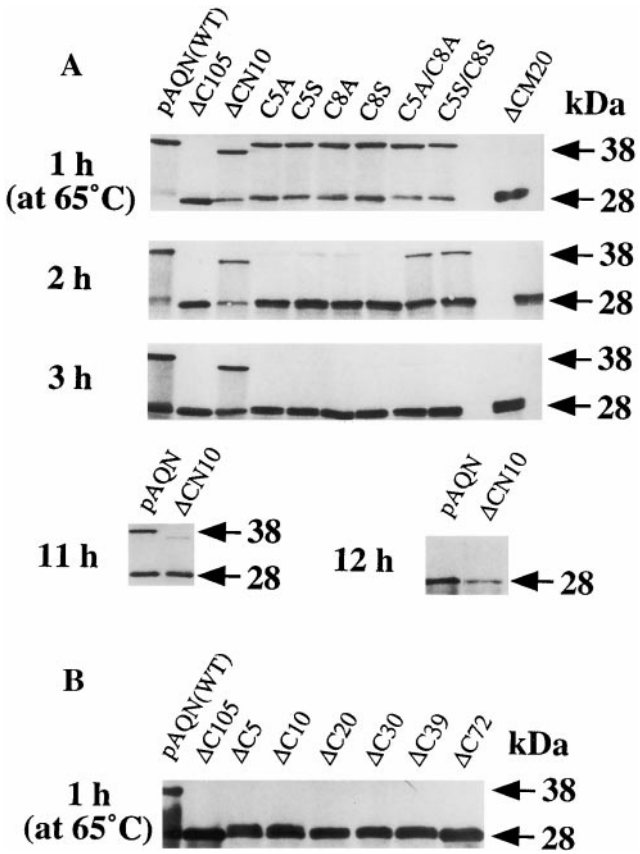

FIG. 2. *In vitro* processing of aqualysin I precursor with mutations in the C-terminal pro-sequence. *E. coli* cells harboring plasmids with mutations in the C-terminal pro-sequence of aqualysin I precursor were cultivated for 2 h at 37°C in modified L-broth medium with 0.05 mM IPTG to induce expression of aqualysin I, collected by centrifugation, disrupted by sonication, and heat-treated at 65°C. At the indicated times, equivalent amounts of samples were analyzed by immunoblotting. (A), substitution and deletion mutations on the N-terminus side of the pro-sequence; (B), deletion mutations on the C-terminus side of the pro-sequence. 38 kDa, aqualysin I with the C-terminal pro-sequence; 28 kDa, mature aqualysin I.

C-terminal pro-sequence mutations to a 28-kDa mature protein was carried out by heat treatment at 65°C (Fig. 2). In the case of the wild-type precursor, the 38-kDa precursor was completely processed to the 28-kDa mature protein by 12 h heat treatment at 65°C. Mutant precursors with substitution or deletion mutations in the C-terminal pro-sequence, with the exception of the Δ CN10 mutation, were processed to the mature protein faster (<3 h) than wild-type precursors. Particularly in the case of deletion mutations, the precursors were more rapidly (<1 h) processed to the mature protein (Fig. 2A, lane Δ CM20, and Fig. 2B). Even at the start of the incubation (0 h), the precursor of Δ CM20 mutation, but not other substitution mutations and wild type, was significantly processed to the 28 kDa mature form (data not shown). These deletion mutations affected translocation of aqualysin I across the cytoplasmic membrane in the *E. coli* expression system (Table II) and its extracellular secretion in *T. thermophilus* expression system (4). These results indicate that such a rapid processing of the C-terminal pro-sequence resulted in the inability of aqualysin I to translocate across the membranes.

Figure 3 shows a strong correlation between the processing rate of the C-terminal pro-sequence (Fig. 2) and proteolytic activation of aqualysin I. The rate of the proteolytic activity in every mutation except the Δ CN10 mutation appeared to be faster compared to wild type, and the proteolytic activation was the fastest in a complete deletion mutation (Δ C105). These results coincide with the previous report (17) that the C-terminal pro-sequence stabilizes an unfolded aqualysin I and prevents folding. From these results, it is reasonable to assume that deletion mutations in the C-terminal pro-sequence resulted in rapid processing of the pro-sequence, thereby promoting the folding of aqualysin I without the C-terminal pro-sequence. As a result, the folded protein failed to translocate across the cytoplasmic membrane.

On the other hand, the Δ CN10 mutant showed the similar pattern as wild-type in *in vitro* processing (Fig. 2) and activation of the protease (Fig. 3). The Δ CN10 mutation lacks ten amino acid residues from the N-terminus of the C-terminal pro-sequence. As shown in Fig. 2, the C-terminal pro-sequence of the mutant is hard to remove. This is likely due to the fact that the mutated portion is directly adjacent to the cleavage site and may influence processing. This mutation did not affect translocation across the cytoplasmic membrane in *E. coli* cells, unlike the other deletion mutations (Table II). However, similar to the other deletion mutations, it diminished the extracellular secretion of the protease in *T. thermophilus* cells (4), suggesting that this mutation might affect the translocation across the outer membrane, but not the inner mem-

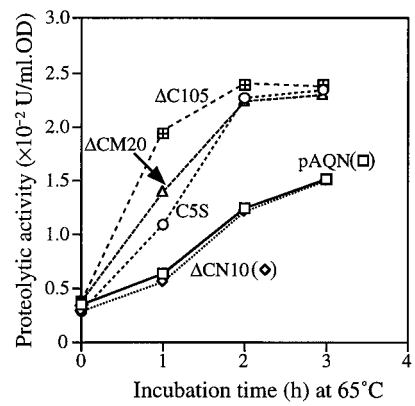

FIG. 3. Proteolytic activation of aqualysin I with various mutations in the C-terminal pro-sequence. *E. coli* cells harboring plasmids with the various mutations in the C-terminal pro-sequence of aqualysin I precursor were cultured, collected, disrupted, and heat-treated at 65°C as shown in the legend of Fig. 2. Aqualysin I activity of each sample was measured at the indicated times.

brane, in *T. thermophilus* cells. Taken together, these results suggest that the C-terminal pro-sequence may play a role in translocation across both the cytoplasmic and outer membranes.

Newly synthesized polypeptides generally have to be retained in a translocation-competent conformation for efficient translocation across the membrane (18). Molecular chaperones are involved in stabilizing newly synthesized proteins and preventing misfolding or aggregation of proteins during extracellular secretion of proteins (19, 20). Our results suggest that the C-terminal pro-sequence of aqualysin I, although it has a signal peptide, is required for the translocation of the precursor across the cytoplasmic and outer membranes. As shown in the previous report (17), the C-terminal pro-sequence acts as an intramolecular chaperone. Similar to molecular chaperones, the C-terminal pro-sequence seems to stabilize the unfolded aqualysin I precursor in the cytoplasm and keep the precursor structure in a translocation-competent conformation, thus facilitating the translocation of aqualysin I across the membranes.

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