Intramolecular Chaperone Activity of the Pro-region of Vibrio cholerae El Tor Cytolysin*

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Vibrio cholerae synthesizes a toxin named El Tor cytolysin/hemolysin, which lyses erythrocytes and other mammalian cells. This toxin is encoded by the hlyA gene and is synthesized as a precursor form, prepro-HlyA. Prepro-HlyA consists of, from the amino terminus of this protein, a signal peptide, a pro-region, and a mature region. The pro-region is cleaved off extracellularly resulting in activation. To analyze the role of the pro-region, we substituted the native hlyA gene with the pro-region-deleted hlyA gene (hlyAΔpro). The hemolytic activity of the mutant organism was markedly decreased; the product of the hlyAΔpro gene, secreted in the periplasm, was degraded. To compare their abilities to form tertiary structure, the purified mature- and pro-HlyA were denatured and then renatured by reducing the concentration of denaturant; the denatured pro-HlyA recovered almost all activity while the mature-HlyA was not renatured. The sequences of the pro-region and a molecular chaperone, Hsp90, were similar. The pro-region expressed in Escherichia coli containing the hlyAΔpro gene increased the cytolytic activity. The purified pro-region peptide also facilitated renaturation of the denatured mature HlyA. These results suggest that the pro-region possibly guides the folding of the cytolysin similar to a molecular chaperone; the pro-region and molecular chaperones share common function and structure.

Vibrio cholerae O1 biotype El Tor, which is the causative agent of the current seventh cholera pandemic, secretes a cytolytic toxin (V. cholerae El Tor cytolysin/hemolysin). The cytolysin lyses erythrocytes and other mammalian cells and exhibits enterotoxicity in experimental diarrhea models (1). Thus, the cytolysin may contribute to the pathogenesis of gastroenteritis caused by V. cholerae strains, especially the strains not producing cholera toxin, the major cause of cholera diarrhea.

El Tor cytolysin/hemolysin is encoded by hlyA and synthesized as an 82-kDa precursor form (prepro-HlyA) (2). The prepro-HlyA consists of, from the amino terminus, a signal peptide (25 residues), a pro-region (132 residues), and a mature region (584 residues) (2). The signal peptide is cleaved during secretion through the bacterial inner membrane, resulting in pro-HlyA. Following secretion through the outer membrane, a 15-kDa pro-region of pro-HlyA is cleaved, generating mature HlyA. Removal of the pro-region to yield mature HlyA results in a more than ten-fold increase in cytolytic activity (2). The role of the pro-region, however, in production and the secretion process is not known.

It is well known that proteases are produced in a precursor form and processed to active enzymes by releasing the pro-region of the precursor protein. Recently, the pro-regions of a few serine proteases, namely, subtilisin E of Bacillus subtilis (3), α-lacto-lytic protease of Lysobacter enzymogenes (4), and carboxypeptidase Y of Saccharomyces cerevisiae (5), have been shown to have a chaperone-like activity with their mature regions in vitro. To distinguish the pro-regions of these proteases from molecular chaperones, they are called intramolecular chaperones; of importance is that these N-terminal peptides possess chaperone-like activity that only functions in folding of the precursor protein of which they are a part (6, 7). The roles of intramolecular chaperones in vitro, where and how they work in the cell, however, are still not well understood. Further, it has been reported that the isolated pro-regions of subtilisin E and α-lacto-lytic protease guide the folding of the mature enzyme intermolecularly as well as intramolecularly (3, 4, 7).

In this paper, we show that the pro-region of El Tor cytolysin works as a chaperone for the cytolysin, a protein that is biologically and physically different from the serine proteases. The pro-region seems to act like a molecular chaperone (e.g. heat shock protein 90 (Hsp90)). Moreover, we present evidence suggesting that the isolated pro-region of HlyA acts like an intramolecular chaperone in facilitating the folding of cytolysin.

EXPERIMENTAL PROCEDURES

Bacterial Strain, Plasmids—Bacterial strains and plasmids used in this study are listed in Table I. V. cholerae N86 was the original strain used in this study. Escherichia coli HB101, SM10pir and BL21(DE3) were used for general manipulation of plasmids, mobilization of plasmid into V. cholerae, and expression of fusion protein, respectively.

Media—Luria-Bertani (LB) broth or LB agar was used for general bacterial growth (8). To observe hemolysis of colonies on agar plates, LB medium containing 1.5% agar and 1.5% (v/v) washed fresh sheep erythrocytes (SB-LBA) were used. For Western blot analysis and pulse-chase experiments, syncaze medium with 3% glycerol (9, 10) and M9 minimal medium with 0.1% casamino acids (Difco) (2) were used, respectively. In the construction of mutant clones through conjugation, thiosulfate citrate bile salt (TCBS) agar medium (Nissui Seiyaku, Tokyo, Japan) was used for counterselection of E. coli.

Chemicals, Recombinant DNA Techniques and Protein Purification—Enzymes and chemicals were purchased from standard commercial sources. Manipulation of DNA was carried out as described previously (8). Site-directed mutagenesis was performed by the method of Kunkel (11, 12). El Tor cytolysin (mature HlyA) was purified from the supernatant of V. cholerae N86, as previously reported (10). Antibodies against purified mature HlyA were affinity purified from rabbit antiserum, as described previously (10). Purification of fusion peptides was performed using pET Expression System 25b (Novagen, Madison, WI).

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of 10 mM phosphate-buffered saline (PBS), pH 7.0. Electrophoresis on a 10% polyacrylamide gel and Western blot analysis were carried out as described previously (2).

Following incubation for 2 h at 37°C, the chloramphenicol-resistant colonies resulting from single-crossover events, were isolated. One of the isolates was cultured in LB broth without antibiotics. Colonies resulting from the second crossover were tested for chloramphenicol sensitivity on LB agar plates with 5 μg/ml of chloramphenicol. Chloramphenicol-resistant colonies were confirmed for the presence of about a 400-base pair deletion by Southern blot hybridization.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting Analysis.** The homologous recombination as described previously (13). Briefly, E. coli strain SM10apir harboring a recombinant plasmid having a mutant gene on a suicide vector, pKY719, was conjugated with V. cholerae. Conjugation was performed on nitrocellulose membranes overlaid on LB agar plates. Following incubation for 2 h at 37°C, the mating bacteria were washed from the membranes and inoculated onto TCBS agar plates. The mixture was then added, followed by further incubation. After 3 min, the precipitated cell pellet was immediately suspended in 20 μl of SDS-polyacrylamide gel electrophoresis sample buffer, heated for 3 min at 95°C, and centrifuged (5,000 g) to remove the supernatant. The precipitate was washed five times with Buffer B, followed by heating at 95°C for 3 min to ensure solubilization, and used as the supernatant sample.

**Pulse-Chase Experiment**—A pulse-chase experiment was carried out basically by the method described previously (2). In brief, 50 μl of an overnight culture in LB broth were inoculated into 10 ml of synace medium (9) supplemented with 3% glycerol in Petri dishes. After a 9-h stationary incubation at 30°C, cells were collected by centrifugation at 5,000 × g for 5 min. The precipitated cell pellet was suspended in 1 ml of 2,000 unit/ml polymyxin B for 30 min at 4°C (14) to stimulate the release of periplasmic proteins. The bacterial cells were removed from the supernatant by centrifugation. The culture supernatant, separated from the cell fraction, was precipitated by addition of 1 ml of 100% (v/v) trichloroacetic acid. Following centrifugation for 15 min at 1,500 × g, the precipitate was suspended in 1 ml of 10 mM phosphate-buffered saline (PBS), pH 7.0. Electrophoresis on a 10% polyacrylamide gel and Western blot analysis were carried out as described previously (2).

**Gene Replacement**—Replacement of a native gene on V. cholerae chromosome with a mutated gene was carried out by double-crossover homologous recombination as described previously (13). Briefly, E. coli strain SM10apir harboring a recombinant plasmid having a mutant gene on a suicide vector, pKY719, was conjugated with V. cholerae. Conjugation was performed on nitrocellulose membranes overlaid on LB agar plates. Following incubation for 2 h at 37°C, the mating bacteria were washed from the membranes and inoculated onto TCBS agar plates. The mixture was then added, followed by further incubation. After 3 min, the precipitated cell pellet was immediately suspended in 20 μl of SDS-polyacrylamide gel electrophoresis sample buffer, heated for 3 min at 95°C, and centrifuged (5,000 × g). 10 μl of the supernatant were diluted with 900 μl of Buffer B (50 mM Tris-HCl, pH 7.5, 0.1% NaN₃, 0.05% bovine serum albumin and 5 mM EDTA), and undissolved materials were removed by centrifugation. The sample was mixed with 30 μl of probe, radiolabeled with [35S]methionine and [35S]cysteine (100 μCi), and incubated for 5 min with a Sepharose CL4B gel coupled with protein A (50% (v/v) suspension) and incubated for 2 h at 0°C. The mixture was centrifuged, and the supernatant was discarded. The precipitate was washed five times with Buffer B, suspended in 20 μl of SDS-polyacrylamide gel electrophoresis sample buffer, heated for 3 min at 95°C, and centrifuged (5,000 × g). 10 μl of the supernatant were applied to SDS-polyacrylamide gels.

**Denaturation and Renaturation Procedure**—Denaturation and rena-
Hemolytic activity of pro- and mature HlyA after denaturation and renaturation

1.25 nmol/ml purified pro- and mature HlyA were mixed into 6 M guanidine HCl and incubated for 2 h at 20°C for denaturation. After incubation, the denatured samples were diluted 60 times with renaturing buffer (10 mM sodium phosphate buffer, 0.15 M NaCl, 0.1% bovine serum albumin, and 5 mM 2-mercaptoethanol, pH 7.0) and incubated for 30 min at 20°C. Samples were then mixed with an equal volume of 100 ng/ml trypsin and incubated at 37°C for 30 min.

| Toxin         | Treatment with guanidine HCl | Hemolytic activity<sup>a</sup> |
|---------------|------------------------------|-------------------------------|
| pro-HlyA      | Treated<sup>b</sup>          | 222 ± 64<sup>d</sup>         |
|               | Untreated<sup>c</sup>        | 232 ± 61                      |
| mature HlyA   | Treated<sup>b</sup>          | <10                           |
|               | Untreated<sup>c</sup>        | 325 ± 116                     |

<sup>a</sup> Hemolytic activity was determined as described under "Experimental Procedures."

<sup>b</sup> Denatured and diluted 60 times.

<sup>c</sup> Control was diluted only, not denatured.

<sup>d</sup> Values (mean ± S.D.) are the results of three experiments.

Pro-region of El Tor Cytolysin Acts Like a Chaperone

by pulse-chase and Western blot analysis. A, pulse-chase measurement of El Tor cytolsin. Revertant (left panel) and N86hlyA<sub>Δpro</sub> (right panel) were labeled for 3 min with [35S]methionine and [35S]cysteine and chased with unlabeled methionine and cysteine for 30 s (lanes 1 and 4), 3 min (lane 2 and 5), and 15 min (lane 3 and 6). Lanes 1–3 are periplasmic fractions. Lanes 4–6 are supernatant fractions of the culture. El Tor cytolsin antigens were precipitated with the affinity-purified antibodies against El Tor cytolsin and electrophoresed. B, Western blot analysis of El Tor cytolsin. Periplasmic fraction (lane 1) and supernatant (lane 2) of revertant (left panel) and N86hlyA<sub>Δpro</sub> (right panel) are shown.
the product of this mutant gene in N86hlyAΔpro. Pulse-chase analysis showed no HlyAΔPro in the culture medium of N86hlyAΔpro although native HlyA of N86-RV was secreted within a few minutes (Fig. 2A). On the other hand, in the periplasmic fraction of N86hlyAΔpro, several 30–40-kDa degradation products, which were not seen in N86-RV, appeared rapidly (Fig. 2A). Western blot analysis gave similar results. HlyAΔPro was not secreted into culture medium although some degradation products were evident (Fig. 2B). These data indicate that the HlyA pro-region is essential for secretion and that HlyAΔPro stays in the periplasmic space where it is degraded.

**Denaturation of Pro- and Mature HlyA**—As misfolded or denatured proteins are considered to be more susceptible to proteases and degraded more easily than folded proteins (20), we suspected that HlyAΔPro might be defective with regard to folding. We compared the renaturation abilities of denatured pro- and mature HlyA proteins. First, to test the influence of denaturant on activities of both toxins, guanidine HCl was added to each toxin. Following incubation for 2 h at 20°C, hemolytic activity was determined. Hemolytic activities of both toxins decreased similarly with increasing concentration of guanidine HCl; both toxins lost almost all in the activities when the concentration of guanidine HCl was 1 M (data not shown).

**Renaturation of Denatured Pro- and Mature HlyA**—To denature the pro- and mature HlyA completely, both toxins were incubated with 6 M guanidine HCl for 2 h. After denaturation, the mixtures were quickly diluted with renaturing buffer. After incubation for 30 min at 20°C, hemolytic activity was determined (Fig. 3). After dilution of guanidine HCl in the samples, the specific activities of the pro-HlyA increased. When the final concentration of guanidine HCl was decreased to 0.1 M, the activity of the denatured pro-HlyA was recovered completely. On the contrary, the activity of denatured mature HlyA was not recovered even when the concentration of denaturant was decreased to 0.1 M.

As trypsin activates pro-HlyA by removal of the pro-region (21), the influence of the denaturation and renaturation on activation was investigated. Pro-HlyA and mature HlyA were denatured in 6 M guanidine HCl. These solutions were then diluted to give a final concentration of guanidine HCl of 0.1 M, and treated with trypsin to activate pro-HlyA. The activity of the renatured and activated pro-HlyA was 992 HLU/nmol, which was comparable with untreated pro-HlyA (232 HLU/nmol) (Table II). However, the recovery of activity with the mature HlyA was poor (<10 HLU/nmol, less than 3% of undenatured control). These results suggest that the mature HlyA is unable to fold itself and that the HlyA pro-region may contribute to the refolding of the denatured toxin.

**Homology between Pro-region and Molecular Chaperones**—Molecular chaperones (22, 23) are considered to mediate the folding of newly synthesized proteins in vivo and are also known to promote folding of denatured proteins in vitro. If the pro-region and molecular chaperones have similar effects, some structural homology among these proteins would be expected. We compared the amino acid sequence of the pro-region with those of chaperones in EBI and SWISS-PROT data bases. A highly homologous region was observed between the pro-region and a heat shock protein, Hsp90, which is a member of a family of molecular chaperones (15) found in *Escherichia coli* from humans (24–26) (Fig. 4). The homologous region found in Hsp90 is highly conserved among the Hsp90 family proteins and are also known to promote folding of denatured proteins in vitro.
The isolation of the Pro-peptide facilitates the formation of denatured mature HlyA in vivo. In this study, we investigated the possible folding ability of the pro-region of El Tor cytolsin (HlyA). The guanidine HCl-denatured pro-HlyA recovered hemolytic activity following dilution of the denaturant; the mature HlyA lacking the pro-region was not activated (Fig. 3 and Table II). This indicates that the pro-region may support the folding of HlyA. The pro-region-deleted HlyA, HlyAPro, was not secreted and was degraded in the periplasmic space (Fig. 2). These results suggest that the degradation of HlyAPro in vitro may be due to its failure to fold in the periplasmic space.

Molecular chaperones (22, 23) are known to enhance the formation of the native conformation of other proteins. In the organelles (e.g. mitochondria and endoplasmic reticulum), molecular chaperones facilitate the folding of proteins exported across membranes. In bacterial cells, although several cytoplasmic molecular chaperones have been found and well characterized (22, 23), few chaperones have been reported in the periplasm (20). In the absence of chaperone, the HlyA pro-region is likely to function in a manner similar to molecular chaperones and be effective in the periplasm. Of interest, V. vulnificus cytolsin, which is evolutionarily close to El Tor cytolsin, does not have a pro-region (27). Why El Tor cytolsin has a special domain for folding remains unclear.

The HlyA pro-region has a domain highly homologous to Hsp90, a family of molecular chaperones that stimulates protein folding (15). This chaperone is widely distributed and found in bacteria from humans (24–26). The region in Hsp90, which is similar to the pro-region of El Tor cytolsin, is one of the most conserved regions among the Hsp90 family proteins (24–26). The pro-region was found to act like a chaperone not only intramolecularly but also intermolecularly. These results suggest that the HlyA pro-region and molecular chaperone Hsp90 may share a common mechanism for supporting protein folding. These findings raise the intriguing possibility that the HlyA pro-region may have structural and functional similarities to molecular chaperones.

Recently, the pro-regions of precursor proteins for a few
serine proteases, subtilisin (3), α-lytic protease (4) and carboxypeptidase Y (5), have been shown to have chaperone-like ability in vitro. To distinguish them from molecular chaperones, these pro-regions are called intramolecular chaperones. All these chaperone-like peptides are part of precursor proteins and the peptides possessing chaperone-like function only for the folding of the precursors (6, 7). All the intramolecular chaperones known so far, including the HlyA pro-region, are found in the N terminus of the precursor proteins.

The pro-regions of subtilisin and α-lytic protease have been reported to act not only as intramolecular chaperones but also like molecular chaperones (3, 4, 7); that is, these pro-regions fold their target peptides when these pro- and the mature regions are expressed in trans. The pro-regions of these proteases have no significant homology to the known molecular chaperones, including Hsp90. The HlyA pro-region has no significant homology with the pro-regions of other proteases either. HlyA pro-region may function in a manner different from the pro-regions of these proteases.

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REFERENCES
1. Ichinose, Y., Yamamoto, K., Nakasone, N., Tanabe, M. J., Takeda, T., Miwatani, T., and Iwanaga, M. (1987) Infect. Immun. 55, 1090–1093
2. Yamamoto, K., Ichinose, Y., Shinagawa, H., Makino, K., Nakata, A., Iwanaga, M., Honda, T., and Miwatani, T. (1990) Infect. Immun. 58, 4106–4116
3. Zhu, X., Ohta, Y., Jordon, F., and Inouye, M. (1989) Nature 339, 483–484
4. Silen, J. L., and Agard, D. A. (1989) Nature 341, 463–464
5. Winther, J. R., and Sorensen, P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9330–9334
6. Inouye, M. (1991) Enzymes 45, 314–321
7. Ohta, Y., Hojo, H., Aimo, S., Kobayashi, T., Zhu, X., Jordon, F., and Inouye, M. (1991) Mol. Microbiol. 5, 1507–1510
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Finkelestein, R. A., Atthasampunna, P., Chulasamaya, M., and Charunmethee, P. (1966) J. Immunol. 96, 440–449
10. Yamamoto, K., Ichinose, Y., Nakasone, N., Tanabe, M., Nagahama, M., Sakurai, J., and Iwanaga, M. (1986) Infect. Immun. 51, 927–931
11. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
12. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
13. Ming, X., Yamamoto, K., and Honda, T. (1994) J. Bacteriol. 176, 4754–4756
14. Hirst, T. R., and Holmgren, J. (1987) J. Bacteriol. 169, 1037–1045
15. Wiech, H., Buchner, J., Zimmermann, R., and Jakob, U. (1992) Nature 358, 169–170
16. Tang, G., Iida, T., Yamamoto, K., and Honda, T. (1994) Infect. Immun. 62, 3299–3304
17. Alm, R. A., Stroeher, U. H., and Manning, P. A. (1988) Mol. Microbiol. 2, 481–488
18. Rader, A. E., and Murphy, J. R. (1988) Infect. Immun. 56, 1414–1419
19. Nagamune, K., Yamamoto, K., and Honda, T. (1995) FEMS Microbiol. Lett. 128, 265–269
20. Wulffing, C., and Pluckthun, A. (1994) Mol. Microbiol. 12, 685–692
21. Nagamune, K., Yamamoto, K., Naka, A., Matsuyama, J., Miwatani, T., and Honda, T. (1996) Infect. Immun. 64, 4655–4658
22. Ellis, R. J., and Hemming, S. M. (1989) Trends Biochem. Sci. 14, 339–342
23. Getchel, M.-J., and Sambrook, J. (1992) Nature 355, 33–45
24. Hickey, E., Brandon, S. E., Smale, G., Lloyd, D., and Weber, L. A. (1989) Mol. Cell. Biol. 9, 2615–2626
25. Bardwell, J. C. A., and Craig, E. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5177–5181
26. Pernolet, C., and Mekalanos, J. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9898–9902
27. Yamamoto, K., Wright, A. C., Kaper, J. B., and Morris, J. G. (1990) Infect. Immun. 58, 2706–2709
28. Miller, V. L., and Mekalanos, J. J. (1988) J. Bacteriol. 170, 2575–2583
29. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89