A Cold Acclimation Protein with Refolding Activity on Frozen Denatured Enzymes

Hidehisa KAWAHARA,1,2 Noriko KODAI,2 Mika OSHIO,1 and Hitoshi Oeba1,2

1Department of Biotechnology, Faculty of Engineering and 2High Technology Research Center, Kansai University, 3-3-3, Yamate-cho, Suita, Osaka 564-8680, Japan

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We found that a cold acclimation protein from an ice-nucleating bacterium, Pantoea ananass KUIN-3, has refolding activity on frozen denatured protein. Based on a SDS-PAGE analysis, we confirmed that the cold shock-treated cells of strain KUIN-3 could produce some cold acclimation proteins that inhibit their synthesis by the addition of chloramphenicol during the cold acclimation. Among such proteins, Hsc25 had refolding activity similar to GroELS. Hsc25 was purified to apparent homogeneity by (NH4)2SO4 precipitation and some chromatographies. The purified Hsc25 was composed of 8 subunits of 25,000 each with a molecular mass of 200,000 and had refolding activity against denatured enzymes, which were denatured by heat-treatment at 100°C, cryopreservation at −20°C, or guanidine hydrochloride, in a manner similar to GroELS. The N-terminal sequence of Hsc25 was Met-Arg-Ala-Ser-Thr-Tyr-His-Ala-Ala-Arg-. Furthermore, Hsc25 had a high level of activity at low temperature (12°C). Also, the dissociation constants, Ko (M) as the binding specificity for enolase, mutarotase, isocitrate dehydrogenase, and lactate dehydrogenase were 1.82 × 10−10, 4.35 × 10−9, 8.98 × 10−12, and 3.05 × 10−11, respectively. The affinity of Hsc25 for frozen denatured enzymes was higher than the affinity for heat denatured enzymes when compared with the affinity of GroEL. These results are the first report on the characterization of a purified chaperon that was induced by cold acclimation.

Key words: cold acclimation protein; chaperone; refolding activity; Pantoea ananass

About 80% of the biosphere on the earth’s surface is 15°C or colder. Microorganisms including bacteria often encounter cold conditions and can exist in extremely cold environments like the Antarctic. In this frozen environment, bacteria are exposed to conditions that necessitate the removal of water in order to maintain the structure and function of the cells. During the removal of water after the temperature shift-down such as freezing at a slow cooling rate, bacteria are able to maintain membrane fluidity and a metabolic constant. The membrane fluidities in various Gram-negative1-3 and Gram-positive4 bacteria are effectively altered by changes in the fatty acid compositions of lipids in the membrane. Also, it has been suggested that trehalose and glycerol are major cryoprotectants for cells exposed to freezing damage, serving to maintain various freezing-labile cytoplasmic enzyme activities.5,6 Simultaneously, due to a decrease in the temperature of the mesophiles7 and psychrophiles,8 the synthesis of various proteins, namely cold shock proteins, are induced in spite of a reduction in the rate of synthesis of most cellular proteins. Many studies of cold shock proteins in Escherichia coli9 and Bacillus subtilis10 have addressed these genes, proteins, and functions.

Ice-nucleating bacteria, which include the genera Pantoea (Erwinia),11 Pseudomonas12,13 and Xanthomonas,14 can nucleate ice formation in super-cooled H2O at −2 to −3°C, and incite frost damage in many crops.15 The ability of ice-nucleating bacteria to survive on the surface of a leaf after freezing and thawing has been shown to be due to ice-nucleating activity after temperature decreases and increases.16 This phenomenon shows a high degree of cryotolerance of the ice-nucleating bacteria. The mechanisms of this cryotolerance have been poorly characterized, except for the association of the ice-nucleating activity. Obata et al. have reported that one of the ice-nucleating bacteria, Pseudomonas fluorescens KUIN-1, could produce COR26 having cryoprotective activity against frozen denatured enzymes during cold acclimation.17 However, this strain had no cold acclimation protein, having a refolding activity similar to the heat shock protein, GroELS.18

This study was done in an effort to discover the properties of the cold acclimation protein in Pantoea ananass KUIN-319 that is caused by cold acclimation, i.e., a shift-down in temperature during a spring
frost. Also, we found that denatured cytoplasmic enzymes after freezing were renatured by Hsc25 having a refolding activity, which was produced by a decrease in temperature. Furthermore, the cold acclimation protein, Hsc25 was purified to apparent homogeneity and the affinity constant of Hsc25 was characterized against various denatured enzymes using the BIACore system.

Materials and Methods

Bacterial strains and growth conditions. The origin and phenotypic characteristics of *Pantoea ananass* (*Erwinia uredovora*) KUIN-3 have been previously described. After strain KUIN-3 was aerobically cultured on Trypticase soy medium (BBL Microbiology System) for 24 h at 18°C, the cell suspension was adjusted to an absorbance of 0.1 at 660 nm with 50 mM potassium phosphate buffer (pH 7.0) and this suspension was then used for inoculation at 1%(v/v) in 100 ml of ice-nucleation medium in a 500-ml shaking flask. Cells were cultured for 48 h at 18 or 30°C. One culture was incubated for 24 h at 30°C, then for another 24 h at 12°C as the cold acclimation treatment. Chloramphenicol (1 mg/ml) was added as required. To purify Hsc25, large cultures (2.5 liters) using a jar fermentor were grown on the ice-nucleating medium with the cold acclimation treatment.

Measurements of cryoprotective and refolding activities. Cryoprotective activity was measured by the method described by Tamiya et al. using the commercial lactate dehydrogenase, LDH, from yeast (Oriental Yeast Co.). Measurement of the refolding activity against LDH, which was denatured by guanidine hydrochloride, freeze-stored at -20°C or heat treated at 100°C for 30 min, was done by the modified method described by Badocoe et al. After the LDH solution was dialyzed overnight against several changes of 0.1 M potassium phosphate buffer (pH 7.0) to remove glycerol, the LDH solution (3 U) was denatured by freeze storage at -20°C for 24 h. Also, the LDH solution was denatured by the addition of guanidine hydrochloride (6 M) in 5 mM β-mercaptoethanol and 50 mM triethanolamine (pH 7.0). The denatured LDH (3 U) was refolded by incubation at 25°C for 30 min. After addition of 10 mM phosphatase buffer (pH 7.0) containing 10 mM ATP-MgCl₂ after the addition of the sample with Hsc25 (80 μg). LDH activities before and after denaturing by the three methods and after refolding were measured by the method of Tamiya et al. Based on the refolding percentage (%) that was defined by the following equation: [Activity (U) after refolding—Activity (U) after denaturing] × 100/Native activity (U)—Activity (U) after denaturing, refolding activity was indicated by the amount (μg protein) of renatured LDH under these conditions. GroEL was purchased from the Wako Chemicals Co. Also, the measurement of cryoprotective activity was done by the modified method described by Obata et al. Isocitrate dehydrogenase from yeast, enolase from yeast, and mutarotase from pig kidney were purchased from the Oriental Yeast Co. Measurements of both enzymes were done by the enzyme coupling method.

Purification of Hsc25 from strain KUIN-3. All steps for the purification were done at 4°C.

1) Preparation of crude extract. The cells were harvested from 10 liters of the cold acclimation culture, washed twice with cold 10 mM potassium phosphate buffer (pH 7.0) by centrifugation at 4,700 × g for 30 min, and then suspended in the same buffer (330 ml) containing 1 mM EDTA and 0.8 mM PMSF as the protease inhibitors. Cells were disrupted with a Branson Sonifier Model 200. The crude extract (3.94 mg/ml, 330 ml) was obtained as a supernatant after ultracentrifugation (Himac CP 8Sβ, Hitach Co., Japan) at 100,000 × g for 1 h.

2) Heat treatment and ammonium sulfate fractionation. The crude extract was treated at 80°C for 10 min, centrifuged at 27,700 × g for 10 min and then subjected to ultrafiltration in a Minitan Ultrafiltration Filter Plate (10,000-molecular weight cutoff, Millipore Co.). The resultant solution (3.42 mg/ml, 330 ml) was fractionated using ammonium sulfate at 40 to 60% saturation, and dialyzed three times for 2 h against the same buffer with protease inhibitors.

3) QA52 cellulose column chromatography. The dialyzed protein solution (13.0 mg/ml, 70 ml) was put on a QA52 cellulose column (Whatmann Co., 5 × 9 cm) equilibrated with the same buffer. After washing, the bound protein was eluted with 660 ml of the same buffer containing NaCl in a linear gradient from 0 to 1.0 M at a flow rate of 2 ml/min. The active fractions were combined, concentrated by ultrafiltration to 4 mg/ml, and dialyzed against the same buffer.

4) Phenyl-TOYOPEARL column chromatography. The active fractions (3.25 mg/ml, 40 ml) after adding ammonium sulfate to provide a 30% saturated solution were put on a phenyl-TOYOPEARL column equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 30% ammonium sulfate saturation. The bound protein was eluted with a decreasing linear gradient of 30 to 0% ammonium sulfate saturation in the same buffer at a flow rate of 2.0 ml/min. The active fractions were combined, then dialyzed against the same buffer.

5) Superdex 200 gel filtration column chromatography. The active fractions (5.59 mg/ml, 9 ml) were put on a Superdex 200 column (1.6 × 60 cm, Amersham Pharmacia Co.) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.15
m NaCl. The active fractions were eluted at a flow rate of 1.0 ml/min, combined, and then dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and rechromatographed on a Superdex 200 column (0.32 × 30 cm) with the SMART™ System (Amersham Pharmacia Co.) equilibrated with the same buffer (0.8 mg/ml, 9 ml). The active fractions were combined, dialyzed against the same buffer, and concentrated by ultrafiltration. This preparation (0.41 mg/ml, 4 ml) was used for the subsequent characterization of the Hsc25 from strain KUIN-3.

Protein concentration. The absorbance at 280 nm was measured to monitor the protein during the chromatographic separation. The protein concentration was measured by the method of Bradford using bovine serum albumin as the standard.

Estimation of molecular weight. The molecular weight of Hsc25 was estimated by gel filtration on a Superdex 200 column (0.32 × 30 cm) by the SMART™ System (Amersham Pharmacia Co.) using 10 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl and was also estimated by SDS-PAGE by the method of Laemmli.

Polyacrylamide gel electrophoresis. SDS-PAGE was done in 12.5% acrylamide gel by the method of Laemmli. Electrophoresis was done at a constant current of 40 mA for 110 min at room temperature. The gel was stained with silver nitrate for the proteins. For the relative molecular weight measurement, phosphorylase b (M, 94 k), bovine serum albumin (M, 67 k), ovalbumin (M, 30 k), soybean trypsin inhibitor (M, 20.1 k) and α-lactalbumin (M, 14.0 k) were used as the standards.

Amino acid composition and sequence analysis. The sample solution (total 1 ml) was lyophilized. The lyophilized sample was hydrolyzed with 5.7 M HCl in a sealed tube at 100°C for 24, 48, or 72 h. The threonine and serine contents were calculated using a standard sample as the value at zero time of hydrolysis. Amino acids were measured by an amino acid analyzer (Tosoh Co., Japan). The amino-terminal amino acids were sequenced with one-half of the lyophilized sample on an Applied Biosystems Protein 476A.

Measurement of affinity constant of Hsc25. The analysis of the association constants of Hsc25 with respect to the denatured enzyme was done using the BIA core system. Immobilizations of each enzyme denatured by the heating or freezing treatments were directly attempted on the dextran matrix of a sensor chip (CM5) surface using the same method. Sample were injected at 30°C with a flow rate of 5 ml/min onto the sensor chip surface on which each denatured enzyme had been immobilized using the 10 mM HEPES buffer (pH 7.4) containing 150 mM KCl, 20 mM MgCl₂, and 2 mM dithiothreitol. The faces of the association and dissociation process were analyzed with BIA-logue kinetics evaluation software as described in the standard method.

Results

Protein inductions of Pantoaea ananass KUIN-3 by cold acclimation and their functions

We examined some of proteins induced by cold acclimation at 12°C for 24 h. Based on the SDS-PAGE analysis, we found that the cells prepared by cold acclimation synthesized some proteins (M, 18 k, 22 k, 25 k), which may be cold acclimation proteins (CAPs) (Fig. 1, lane 5). The syntheses of these proteins were inhibited by the addition of chloramphenicol during the cold acclimation treatment (lane 4), and stopped after culturing at 30°C (lane 2) and at 18°C (lane 3). One of these CAPs, the cryoprotective protein (CRP), might be related to the cryotolerance of the cells in P. fluorescens KUIN-1, the cytoplasmic protein fractions preparing from cold-acclimated cells were examined for both cryoprotectively and refolding activities. These fractions indicated only a high level of refolding activity against the denatured LDH. Also, the ice-nucleating activity of the cell was activated by the cold acclimation treatment, whereas this cell had a low level of cryoprotective activity and no antifreeze activity. In conclusion, among the three main CAPs, CAP with M, 25 k that was called as Hsc25 had refolding activity as a chape-

![Fig. 1. SDS-PAGE Analysis of Endogenous Proteins of Cells Grown under Various Conditions.](image-url)
Purification and characterization of Hsc25

The heat shock protein, GroELS, found in E. coli\(^{22}\) had refolding activity against various enzymes that were denatured by heat shock treatment and consequently was classified as a chaperonin. Therefore, Hsc25 was purified to apparent homogeneity by various chromatographies described in the Materials and Methods Section A. A summary of this purification step is shown in Table 1, and 1.62 mg of Hsc25, the purity of which was the refolding percentage of 95%, was obtained from 1,300 mg of protein from the crude extract. As shown in Fig. 2, Hsc25 migrated as a single band. The relative molecular weight of Hsc25 was estimated to be 25,000 by SDS-PAGE (Fig. 2) and to be 200,000 by gel filtration. These results indicated that Hsc25 was composed of 8 subunits of 25,000 each with a molecular weight of approximately 200,000. The refolding activities, which were indicated by the amount of renatured LDH, of the purified Hsc25 against denatured LDH on frozen or guanidine hydrochloride treatments are shown in Table 2 in view of the natural refolding. The amount of renatured LDH without ATP-Mg\(^{2+}\) were 4.4 and 1.0 \mu g, respectively. In contrast, this activity in the presence of 10 mM ATP was equal to that without ATP, while it was stimulated up to 10 \mu g by the addition of 10 mM ATP-Mg\(^{2+}\). GroES from E. coli expressed ATPase activity to release the refolded enzyme in the presence of ATP-Mg\(^{2+}\).\(^{22}\) It seems that Hsc25 may act as a chaperon with the specificity for some enzymes denatured by freezing. We found that Hsc25 had the ATPase activity (7.83 U/mg) and was combined with two ATP molecule per one oligomer by the measurement of ATP content in the native Hsc25 with a molecular weight of 200,000. The complex of Hsc25 and ATP was formed before it bound to denatured enzymes as the substrate. Next, the effects of temperature on the refolding activity of Hsc25 were examined. (Fig. 3) In the absence of ATP-Mg\(^{2+}\), Hsc25 had refolding activity below
30°C, while the refolding activity of Hsc25 was stimulated at all temperatures by the addition of ATP-Mg²⁺, including at 37°C.

Amino acid composition and N-terminal amino acid sequence
The amino acid composition of the purified Hsc25 was examined. His, Lys, Val, and Ser residues were abundant in the Hsc25 molecule and the contents of hydrophilic amino acids were higher than that of hydrophobic amino acids. Hsc25 has a high proportion of hydrophobic amino acid residues with an OMH value below -0.6. The N-terminal sequence of Hsc25 was analyzed to the 10th residue, Met-Arg-Ala-Thr-Tyr-His-Ala-Ala-Arg-. A BLAST computer comparison of the proteins in the Protein Identification Resource did not discover any peptides with a similar sequence.

Affinity constant of Hsc25 for some denatured enzymes
The affinity constant of Hsc25 for some denatured enzymes was measured using the BIAcore system. When Hsc25 was injected over the sensor chip with immobilized frozen LDH, the RU level increased up to about 1000 RU. This change in RU was not observed when the same concentration of BSA was injected. As shown in Table 3, Hsc25 has a broad specificity, indicating a Kᵦ from 8.91 × 10⁻¹² to 1.82 × 10⁻¹⁰ for various denatured enzymes that were cold-labile, key enzymes for the biogenic metabolism. Moreover, Hsc25 could not bind to non-denatured LDH so that we found that Hsc25 had a broad substrate specificity for various dentured enzymes and could preferentially bind to the denatured enzyme. Also, Hsc25 could refold freeze-denatured enzymes than Gdn-HCl denatured enzymes on all enzymes as shown in Table 3. As the Kᵦ shows the dissociation constant, the binding affinity of Hsc25 for various denatured enzymes increases with decreases in this value. The Kᵦ value between GroEL and denatured LDH was very small. The Kᵦ values of Hsc25 under various conditions were examined using BIAcore as shown in Table 4. In spite of the presence of ATP-Mg²⁺, the affinity of Hsc25 for the heated LDH was lower than that of GroEL. On the other hand, the affinity of Hsc25 (3.05 × 10⁻¹¹) for the frozen LDH in the absence of ATP-Mg²⁺ was slightly higher than that of GroEL (6.78 × 10⁻¹¹). This result indicates that Hsc25 may in vivo act mainly on the freeze-denatured enzymes considering the property that Hsc25 must acclim in the cold condition. Also, the affinity constant of Hsc25 was markedly increased by the addition of ATP-Mg²⁺ due to accelerating the dissociation of Hsc25 from renatured LDH similar to the change in the Kᵦ value of GroEL. Moreover, the addition of ADP-Mg²⁺ could not stimulate the dissociation of Hsc25 from renatured LDH. In view of the kinetics analysis of interaction between GroEL and reduced α-lactalbumin described by Murai et al., this dissociation can be an important factor for the refolding activities of various chaperons. We assumed that the refolding mechanism of Hsc25 may be similar to that of GroEL.

Discussion
Most of the ice-nucleating bacteria have been isolated from the leaves of plants damaged due to frost injury and sensitive to cold conditions. These frost injuries were caused by ice-nucleating activity, which catalyzed ice formation at temperatures as high as -2 ~ -3°C without a supercooling point. After freezing and thawing under frost injury conditions, these bacteria were the major types among the epiphytic bacterial populations on the leaf surfaces. Ice-nucleating bacteria seem to have some unique features for surviving freezing temperatures. We predicted one mechanism that could protect some intracellular components including metabolic enzymes against freezing condition, except for the mechanism for minimizing freezing injury on the cell surface due to extracellular ice formation. Obata et al. have reported that an ice-nucleating bacterium, Pseudomonas fluorescens KUIN-1 could produce one cryoprotective protein, COR26 by cold acclima-

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<th>Enzyme</th>
<th>Affinity constant, Kᵦ (M)</th>
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<tr>
<td>Enolase</td>
<td>1.48 × 10⁻⁶</td>
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<td>Mutarotate</td>
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<td>iCDH²</td>
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<td>LDH²</td>
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<th>Enzyme</th>
<th>Chaperone</th>
<th>Affinity constant, Kᵦ (M)</th>
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<tr>
<td>Heated LDH³</td>
<td>Hsc25</td>
<td>1.05 × 10⁻¹⁰</td>
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<tr>
<td>GroEL</td>
<td>7.70 × 10⁻¹¹</td>
<td>9.02 × 10⁻⁹</td>
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<td>Frosed LDH³</td>
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<tr>
<td>GroEL</td>
<td>6.78 × 10⁻¹⁲</td>
<td>6.93 × 10⁻⁹</td>
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³ The heated LDH was prepared as the denatured enzyme by heating at 100°C for 30 min.
² The frozen LDH was prepared as the denatured enzyme by freezing at -20°C for 24 h.
Cold Acclimation Protein with Refolding Activity

Assuming the function of Hsc25 in vivo, those were examined the extent of the freeze sensitivities of some enzymes from strain KUIN-3. The high affinity constant of Hsc25 to denatured iCDH has consequently been reflected by iCDH from strain KUIN-3 being extremely denatured by the freezing treatment. The function of Hsc25 under low temperature might have an important role of protection against freeze denatured enzymes in the cytoplasmic space. Also, it remains unknown that Hsc25 in vivo acts on the cytoplasmic enzymes denatured by freezing in combination with other cold acclimation proteins. As Hsc25 could not bind to ADP and GTP, this refolding mechanism is the focus of intense research. However, this is the first report of a cold acclimation protein having a refolding activity against freeze-denatured enzymes. The structure of Hsc25 and its presence in other bacteria are currently being studied.

References

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