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Renaturation of Lysozyme with a Protein Disulfide Isomerase Chaperone Results in Enzyme Super Activity

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When the oxidative refolding of lysozyme (Lyzm) was carried out in the presence of protein disulfide isomerase (PDI) an increased refolding rate and a recovered activity exceeding 100% were reproducibly observed. The origin of this excess activity was investigated by HPLC, SDS–PAGE, and mass spectrometry and assessed using an assay for Lyzm activity. The refolding of Lyzm was achieved through the formation of PDI-Lyzm intermediates and the excess activity was derived from the nascent lysozyme released from these complexes. The released lysozyme exhibited a higher molecular activity than observed for the native protein.

[**Key words:** chaperoned lysozyme, lysozyme refolding, protein disulfide isomerase, super activity]

The oxidative refolding of fully reduced hen egg white lysozyme (r-Lyzm) has been established (1–4). Using a solution having the appropriate molar ratio of urea to LiCl in the refolding media with redox reagents such as reduced glutathione (GSH) and oxidized glutathione (GSSG), r-Lyzm is almost completely renatured (1, 2). Recently, it was reported that when protein disulfide isomerase (PDI) was applied to renaturation solution, the refolding rate was reproducibly increased and the recovered enzymatic activity always exceeded 100% (5). The reasons behind the excess enzymatic activity might provide valuable insight that might be applied to the renaturation processes of other enzymes.

We determined that the increased activity was not attributed to (i) impurities in the starting material, (ii) a suppression of protein aggregation, or (iii) a cooperative effect of PDI on the Lyzm activity assay (5). The excess activity was only apparent when the activity of renatured Lyzm was measured in the presence of PDI. The effect lasted for 2 to 3 d and then decreased gradually. After a week or more, the activity settled at the usual 100% activity as was observed when the enzyme was refolded without PDI. This implied that the renaturation procedure in the presence of PDI caused the formation of a temporary Lyzm folding intermediate having a super activity or an activity higher than that of native Lyzm (n-Lyzm).

Others reported that PDI functions as a molecular chaperone (6, 7) in addition to a disulfide-isomerase (8, 9). But PDI may have other abilities that have yet to be discovered. As yet, the details of both the chaperone and anti-aggregation functions of PDI remain to be determined. The goal of this work was to identify the nature of the PDI-Lyzm complex and determine the source of the super activity observed.

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MATERIALS AND METHODS

Preparation of Lyzm, r-Lyzm, and PDI Lyzm and the dried *Micrococcus lysodeikticus* that served as the Lyzm substrate were purchased from Sigma. r-Lyzm was prepared as described previously (1). Lyzm (100 mg/10 ml) was prepared in a 0.1 M Tris–HCl buffer containing 10 M urea and 100 mg dithiothreitol (DTT) at pH 8.0 and was incubated for 2 h at 40°C. The solution was then acidified to pH 3 with 1 M HCl to stop the reduction. The mixtures were applied to a column (2.2×50 cm) composed of Sephadex G-25 (coarse grade), which was equilibrated and eluted with 0.1 M acetic acid. The fractions containing r-Lyzm were lyophilized and stored at –25°C. The concentrations of Lyzm and r-Lyzm were determined spectrophotometrically and calculated using their molar extinction coefficients, 37,600 and 33,890 M^{-1} cm^{-1}, respectively (10).

Recombinant fungal PDI (*M*r 53,100) was used for our experiments. Stable noncovalent PDI dimers are formed except under extreme conditions (11). Although this PDI has a more acidic pI than that of yeast or bovine PDI, there is essentially no difference in their function (11). Fungal PDI was prepared using a high-level *Bacillus brevis* secretion system as was described previously (9, 11, 12).

Activity assay An *M. lysodeikticus* cell wall suspension (0.25 mg/ml, with an initial turbidity of 1.0 absorbance unit at 450 nm) was prepared in 50 mM sodium phosphate buffer at pH 6.2 (13). The Lyzm activity was determined from the decrease in the turbidity of the solution with time. The decrease in turbidity after adding 10 to 100 µl of native Lyzm (n-Lyzm) or renatured Lyzm to 3.0 ml of the suspension was measured at 30°C.

Refolding procedure The degree of the excess activity depended on the concentrations of both Lyzm and PDI. When a low Lyzm concentration and a large molar ratio of PDI to Lyzm, *i.e.*, when the concentrations of Lyzm and PDI were 5 µM and 62 µM, respectively, the recovered activity was able to reach up to 170%. In order to have enough Lyzm for analysis, the Lyzm concentration was set to 10 µM. The recovered activity was recorded as a percentage relative to the activity measured using an n-Lyzm solution containing the protein at the same concentration as in the refolding solution. The refolding procedures employed were the same as

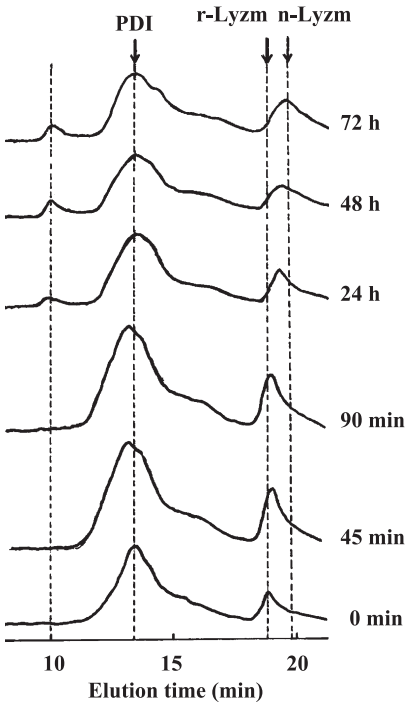

FIG. 1. Time course of refolding as traced by gel-HPLC. Arrows indicate the elution times for protein disulfide isomerase (PDI, dimer), fully reduced lysozyme (r-Lyzm), and native lysozyme (n-Lyzm). The column was of TSKgel G3000SWXL (0.78×30 cm; Tosoh, Tokyo). The elution was carried out by 0.5 M Tris–HCl (pH 8.0) containing 2.5 M urea and 0.5 M LiCl at a flow rate of 1 ml/min. The results were detected by UV at 280 nm.

those reported previously (5). Lyophilized r-Lyzm was prepared by the method described above and was dissolved in Tris–HCl buffer (pH 8.0) containing 6 M guanidinium chloride for a final concentration of 1 mM. This r-Lyzm solution was diluted to 10 μM and mixed into a solution containing 2.5 M urea and 1.5 M LiCl. Then PDI and DTT were added at concentrations of 62 μM and 5 μM, respectively. The refolding reaction was initiated by introducing GSH and GSSG at concentrations of 3 mM and 0.3 mM, respectively.

Analyses of the refolding solution and PDI-Lyzm complex To analyze the refolding solution, gel-HPLC and reverse phase-HPLC (RP-HPLC) were employed. For analyses of the fractions separated by the column used for the gel-HPLC analysis as described above, RP-HPLC, SDS–PAGE, and TOF-MS were adopted to study the features of complexes of PDI with Lyzm and to examine the features of the Lyzm form having super activity. The gel and RP columns used were TSKgel G3000SWXL (0.78×30 cm; Tosoh, Tokyo) and Protein C4 (Vydac, 0.46×30 cm; The separations group, Hesperia, CA, USA), respectively. The eluent for gel-HPLC was placed in 0.5 M Tris–HCl buffer (pH 8.0) containing 2.5 M urea and 0.5 M LiCl. These conditions were analogous to those of the refolding buffer to ensure that the complexes in the refolding solution remained intact during analysis. The flow rate was 1 ml/min except during fractionation. The RP-HPLC elution was carried out by increasing the acetonitrile concentration in 0.1% trifluoroacetic acid linearly from 33% to 67% for 45 min at a flow rate of 1 ml/min. Eluates were detected by UV at 280 and 220 nm for gel- and RP-HPLC, respectively. The polyacrylamide gels used for SDS–PAGE were Peptide-PAGE mini (TEFCO, Tokyo) gels and the mass analysis was done on an Ultraflex apparatus (Bruker Daltonics, Billerica, MA, USA).

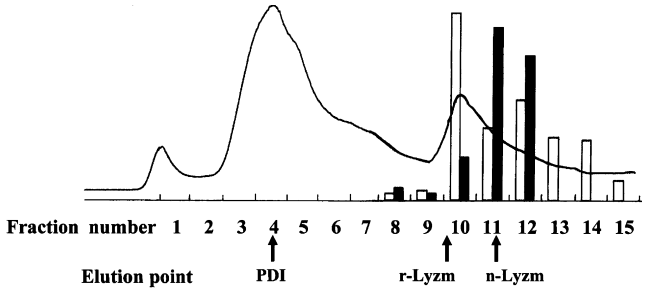

FIG. 2. The relative activity of each fraction obtained by gel-HPLC for the refolding solutions after 24 h and 9 d. Open and solid bars indicate the relative activity of each fraction after 24 h and 9 d, respectively. The relative activity is the value obtained when comparing the activities of the individual fractions. Conditions were the same as those described in Fig. 1 except for the flow rate, which was 0.6 ml/min.

RESULTS AND DISCUSSION

Changes in the renaturation solution with refolding time The recovered activity measured in the refolding system described in the Materials and Methods exceeded 100% after 1 h and reached approximately 130% after 1.5 h. This activity level was maintained for several days, then decreased gradually, reaching a standard 100% activity 9 d later.

Figure 1 shows the time course for refolding as described by the gel-HPLC elution profiles. Arrows indicate the elution position for PDI (dimer), r-Lyzm, and n-Lyzm. A relatively extensive range termed tentatively as the Lyzm-region (18–21 min), indicated where various Lyzm forms, including r-Lyzm and n-Lyzm, may elute. It was evident that the top of the broad peak emerging in the Lyzm-region began to shift to the right and the peak at about 13 min shifted to the left after 45 min. The new peak emerged at approximately 10 min after 24 h of refolding. We suspected this feature was an indicator that PDI formed a complex of some kind with Lyzm. This elution position corresponded to a molecular weight estimated as around 4×10^5 from the calibration curve for this column.

Existence of a PDI-Lyzm complex Figure 2 shows the gel-HPLC elution profile traced by the solution after 24 h of refolding. The fraction numbers are shown along the abscissa. The fractionation took place at a flow rate of 0.6 ml/min. The elution positions for the protein standards are also illustrated. Repeating this process enriched the fraction.

If present, the PDI-Lyzm complex should elute far from the Lyzm-region in gel-HPLC tracing. The results of SDS–PAGE analysis for fraction one (F1) and of RP-HPLC findings for fraction four are shown in Figs. 3 and 4, respectively. The right lane in Fig. 3 was for the standard samples, and their corresponding elution profile is shown in Fig. 4. It was noted that the major PDI band or peak that was observed by SDS–PAGE or during RP-HPLC analysis, respectively, corresponded to the monomeric form. But the PDI peak in gel-HPLC reflected that of the dimer (*Mr* 106,200). The elution time for the PDI monomer by RP-HPLC was about 17 min. The enriched PDI solution of fraction four also revealed the PDI dimer (PDI_2) at around 30 min. Figure 3