Journal of Biochemistry and Molecular Biology, Vol. 34, No. 2, March 2001, pp. 102-108

JOURNAL OF
Biochemistry
and
Molecular Biology

Characterization of Protein Disulfide Isomerase during Lactoferrin Polypeptide Structural Maturation in the Endoplasmic Reticulum

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Received 22 August 2000, Accepted 28 November 2000

A time-dependent folding process was used to determine whether or not protein disulfide isomerase (PDI) plays an important role in the maturation of nascent lactoferrin polypeptides. Interaction between lactoferrin and PDI was analyzed according to the co-immunoprecipitation of the two proteins. The results indicate that lactoferrin folding requires a significant interaction with PDI and its binding is relatively brief compared to other nascent polypeptides. The amount of lactoferrin interacting with PDI increases up to half a minute and sharply decreases beyond this time point. During the refolding process that follows reduction by DTT, lactoferrin polypeptides heavily interact with PDI and the interaction period was extended compared to the normal folding process. In terms of the temperature effect on PDI-lactoferrin interaction, PDI binds to lactoferrin polypeptides longer at a lower temperature (here, 25°C) than 37°C. The lactoferrin-PDI interaction was also studied *in vitro*. According to the *in vitro* experiment data, PDI was still functional in cell lysates assisting lactoferrin folding into the mature form. PDI interacts with lactoferrin polypeptides for an extended period during the folding *in vitro*. During the refolding process *in vitro*, intermolecular aggregates and refolding oligomers matured into a functional form after PDI binds to the lactoferrin. These results suggest that PDI provides a prolonged chaperoning activity in the refolding processes and that there appears to be a greater requirement for PDI chaperone activity in the refolding of lactoferrin polypeptides.

Keywords: Lactoferrin, Protein folding, Molecular chaperones, PDI, DTT.

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Introduction

During the maturation of the protein structure in the endoplasmic reticulum (ER), secretory polypeptides undergo the secondary structure formation to reach the final structure. The first priority toward structural maturity is to completely correct disulfide bonds between cysteine counterparts (Craig, 1993; Gunther *et al.*, 1993). The secondary structure formed by disulfide bonds plays an important role in the final structure. Under the highly oxidized environment in the ER, however, disulfide bonds often tend to be formed in a random fashion that interferes with maturation into the functional form (Walsh and Smith, 1993; Lee and Pedersen, 1995).

In the ER, protein disulfide isomerase (PDI) is a molecular chaperone that plays a critical role in assisting the nascent polypeptides assembly into the correct protein structure. PDI is responsible for helping cysteine residues to pair correctly, thus, forming correct disulfide bonds. PDI is believed to participate in the quality control system in the ER. In addition, ERp 60 (a PDI homolog) selectively digests polypeptides that are unable to reach the functional form. Thus, it helps in recycling amino acids in the ER (Klausner and Sitia, 1990; Melinick and Argon, 1993).

Because the subject of this study is complicated, the result of our efforts is insufficient in identifying the quality control function that occurs in the ER. For the study, we will use lactoferrin (an iron ion transport protein), whose structure among secretory proteins has been extensively studied. Lactoferrin is a monomeric glycoprotein with a molecular mass of approximately 80 kDa. The protein is made up of 32 cysteines consisting of approximately 4% of the total 727 amino acids.

The effort to overproduce useful proteins like lactoferrin has encountered many problems. One of the reasons may be that polypeptides expressed in heterogeneous hosts are often subjected to discrimination by the hosts. Many heterogeneous proteins form insoluble bodies, or undergo digestion by

cellular proteases. It is unclear why lactoferrin is not easily overproduced in foreign hosts. Elucidation of the mechanism of the formation of insoluble protein aggregates is essential in resolving the problems. In this study, the effects of various types of biomolecules on the aggregation of denatured proteins were investigated.

Recently, it has been proposed that expressed lactoferrin polypeptides might have a tendency for misfolding, because of the unusual number of cysteine residues. The misfolded lactoferrin polypeptides are to be digested by the quality control system, even after successful translation. Thus, this study attempts to characterize the interaction between PDI and lactoferrin polypeptides during structural maturation; and to provide a solution for the overproduction of lactoferrin in economically useful hosts.

Materials and Method

This experiment was performed using the mammary gland tissue isolated from a rat of 12-16 weeks. The tissue was labeled using [^{35}S]methionine/cysteine under the discriminative conditions in time, temperature, and reducing conditions. After sonication at 4°C, labeled proteins were isolated from the cleared cell lysates.

Cell labeling and immunoprecipitations For a pulse-chase analysis, fresh mammary gland tissue was incubated with ^{35}S methionine/cysteine with or without the presence of 2-5 mM DTT in the DMEM. The efficiency of the radio-labeling in the presence of DTT was approximately 30% lower than in the DTT-free solution. This low labeling efficiency problem was solved by long-term starvation of fresh tissues at a low temperature (20°C). As the result of labeling in the solution with DTT, the protein folding process could be reversed without any major problem to the cell activity within 3 to 5 min (a relatively short period of time) (Freedman, 1989; Callea *et al.*, 1992). Labeled lactoferrins were immunoprecipitated by an anti-human lactoferrin antibody (Sigma Chemical, Saint Louis, USA) in a RIPA buffer according to Lee (1998). The PDI or PDI-lactoferrin complex was immunoprecipitated by an antibody against PDI (a generous gift of Dr. P. Kim, Harvard Medical School).

PDI-lactoferrin interaction assay In order to investigate the PDI-lactoferrin interaction during the folding process, lactoferrin was immunoprecipitated by an antibody against PDI in the lysates that were prepared from cells, which were chased for 0-120 seconds. The precipitated lactoferrin was electrophoresed on non-denaturing gels and analyzed according to the electrophoretic mobility. The mammary gland tissue was also labeled overnight to visualize PDI, which were co-immunoprecipitated with lactoferrin.

In order to investigate the PDI-lactoferrin interaction during the lactoferrin refolding, the tissue was treated with DTT. Washing the tissue copiously with a culture media without DTT restored protein folding. During refolding, the interaction between PDI and lactoferrin was studied via co-immunoprecipitation of the two proteins by an antisera against PDI. During the process of protein refolding, PDI associated with its target protein and played a role in the reaction. Depending on the co-immunoprecipitation with the PDI-antibody (cross-linking agent) disuccinimidyl tartrate (DST; Sigma Chemicals) was utilized to preserve the PDI-lactoferrin complex, especially when the interaction was tested *in vitro*.

Temperature effect assay According to recent studies, a low temperature exerts a positive influence in exporting nascent polypeptides from the ER (Adkinson *et al.*, 1990; Kim and Arvan, 1995). Indeed, the export of proteins from the ER is facilitated at a lower temperature (14, 25°C) compared to 37°C. It has been confirmed that mature proteins were discovered partially on the cell surface. It indicates that the low temperatures facilitate the folding and correct the inadequate conformation of mutant polypeptides. Using the methods indicated above, we identified the relation between the temperature and lactoferrin folding. The temperature effect on the PDI-lactoferrin interaction was investigated according to the amount co-immunoprecipitated by the antisera against PDI or lactoferrin.

Test the maturity of protein folding To estimate the process of lactoferrin folding, labeled lactoferrin was isolated at each time point. The incubation was terminated by adding IAA (Iodoacetamide, Sigma Chemical) after incubation with 5 mM DTT for 1 h. Immediately after the time points, DTT was neutralized by IAA, so that the protein conformation treated by restricted DTT can be preserved in each aiming time (Sawyer *et al.*, 1994; Losch and Koch-Brabdt, 1995). Therefore, it can be visualized by the decrease of the rate of movement on a non-denaturing gel. This experiment utilized a cross-linking agent, such as DST, after cell culture to preserve a potential PDI-lactoferrin complex (Alberni *et al.*, 1990; Flynn *et al.*, 1991). For the preparation with DST, lactoferrin was stabilized by adding 0.2 M sucrose. DST was then delivered at 4 mM in a 10 mM sodium phosphate buffer (Lee and Pedersen, 1995).

Results

This study characterized the structural maturation process for nascent lactoferrin polypeptide by the pulse-chase procedure. PDI, which is known to be important in the early stage of protein folding, was the major target in this experiment. The conditions included the reducing or oxidizing status, as well as variable temperatures. The study also investigated the lactoferrin-PDI interaction during refolding of lactoferrin under reducing conditions generated by the addition of DTT.

Rapid binding of PDI with nascent lactoferrin polypeptides When lactoferrin were immunoprecipitated by an antibody against PDI, lactoferrin polypeptides were shown to co-precipitate in the lysates prepared from cells, which were chased for 0-120 seconds. The lactoferrin-PDI interaction appears to reach a peak at approximately 1/2 minute following completion of nascent lactoferrin polypeptides (Fig. 1). This may be unusual since the majority of nascent polypeptides spend at least a few minutes until the high level of interaction with other molecular chaperones (Kim and Arvan, 1995). This result also indicates that the lactoferrin protein folding needs a rapid interaction with PDI

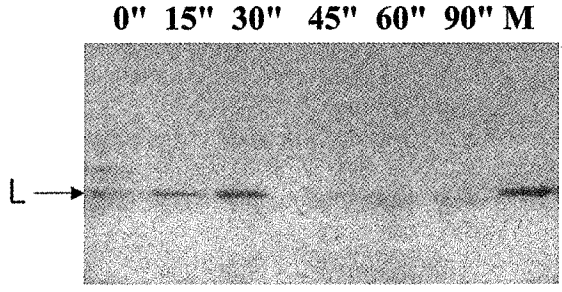

Fig. 1. Immunoprecipitation of lactoferrin by antisera against PDI. Lactoferrin polypeptides (**L**) were immunoprecipitated from the cell lysates following pulse-label and chase. The time points indicate the length of the chase period. The amount of lactoferrin precipitant, pulled down with PDI, culminated at 30 seconds of the chase. After this period, the lactoferrin precipitant drastically leveled off. **M**: Size marker for lactoferrin.

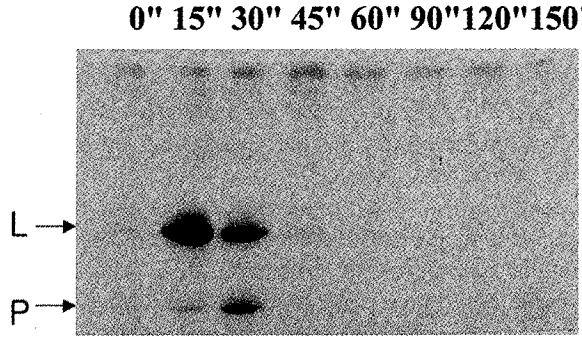

Fig. 2. Immunoprecipitation of PDI following overnight incubation. To recognize PDI interacting with nascent lactoferrin polypeptides, the mammary gland tissue was labeled overnight. PDI were then immunoprecipitated by an antisera against PDI. Lactoferrin (**L**) were co-immunoprecipitated with PDI (**P**). The amount of lactoferrin interacting with PDI increased up to 30" and sharply decreased beyond that time point. Lactoferrin polypeptides appear to interact with PDI for relatively short lengths of time during the nascent peptide folding.

for its structural maturation process. In terms of the duration for binding, the majority of polypeptides release PDI approximately 1 minute after the initial interaction when PDI is detected. Together, the results imply that lactoferrin folding requires an intensive binding of PDI; and its binding is relatively brief compared to other nascent polypeptides (Melinick *et al.*, 1994; Kim and Arvan, 1995).

An additional experiment was performed to confirm whether PDI indeed participates in the lactoferrin folding (Fig. 2). Contrary to Fig. 1, co-precipitation of lactoferrin and PDI was confirmed. As presented in Fig. 1, PDI precipitated with lactoferrin by antisera against lactoferrin. The interaction between the two proteins appears to culminate at 1/2 minute with chase. After that period, PDI was not found to be associated with lactoferrin in the precipitant. This result corresponds with that presented in Fig. 1 and implies that lactoferrin polypeptides require unusual aids of PDI within a relatively short period of time. Based upon these of results, PDI is believed to play an important role in the nascent lactoferrin protein folding. Interaction between lactoferrin and PDI appear to maximize at approximately 30 seconds following polypeptide formation and collapses rapidly after that time point. Lactoferrin were co-immunoprecipitated with PDI. The amount of lactoferrin interacting with PDI increased up to 30 seconds and sharply leveled off after that time point.

Delayed structural maturation keep PDI interacting with lactoferrin polypeptides Attempts were made to recognize potential lactoferrin folding intermediates via the refolding process. The mammary gland tissue was incubated in the presence of DTT. Lactoferrin polypeptide was subjected to reducing conditions, thus, its structural maturation was delayed while lactoferrin was pulse labeled in the presence of DTT. DTT appeared to retard the structural maturation of the lactoferrin polypeptide. Various folding intermediates, and the mature folding form, appeared on the non-denaturing PAGE. Based upon the mobility, transient oligomers (rather than folding intermediates) were prevalent in the refolding process when chase in the DTT-free media began. The lane at the left edge represents lactoferrin polypeptides precipitated by antisera against lactoferrin immediately following incubation. The lane at the right edge depicts the distribution of the lactoferrin folding form at 4 minutes. The lactoferrin oligomers were subjected to the correct folding activity by molecular chaperones during DTT-free incubation. In this experiment, a mature lactoferrin folding form prevailed at 150" of DTT- free incubation.

Fig. 3 shows that denatured lactoferrin polypeptides refolded as time elapsed. As the cellular concentration of DTT decreased, unfolded lactoferrin polypeptides refolded in a time-dependent manner. The extent of refolding was visualized according to the difference in mobility on a non-denaturing gel. The lane at the far left represents lactoferrin polypeptides isolated immediately after incubation with DTT; whereas, the majority of the unfolded lactoferrin was on the verge of structural maturation during the course of the DTT-free incubation. Indeed, the ratio between the matured form and folding intermediates increased as the DTT-free incubation period elapsed. At the early stage of the refolding process, the presence of DTT, although small in quantity, still retarded refolding of lactoferrin polypeptides; and the majority of lactoferrin polypeptides were found as oligomers. Structurally matured lactoferrin were subjected to DTT treatment following an overnight labeling of the cell. As in Fig. 3, correct folding resumed when DTT was diluted in the incubation media. The lactoferrin-PDI interaction was assayed by co-precipitation of the two factors by the antibody against lactoferrin. Denatured lactoferrin appeared to be bound heavily with PDI. Denatured lactoferrin polypeptides showed a spontaneous aggregation following the addition of IAA; thus, causing almost instant aggregation of the denatured

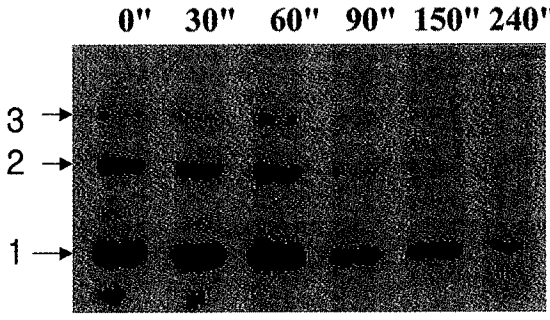

Fig. 3. Refolding of lactoferrin polypeptides under reducing conditions. Lactoferrin was pulse labeled in the presence of 2 mM DTT. During the refolding process, lactoferrin polypeptides formed various multimers. Various refolding oligomers (**2, 3**) and mature folding form (**1**) were separated on a 5% non-denaturing PAGE. The refolding oligomers were prevalent when the DTT-free chase began. The lane at the left edge represents lactoferrin polypeptides precipitated by a lactoferrin antibody immediately after a 5 minute incubation. The lane at the right edge refers to the distribution of the lactoferrin folding form at 4 minutes. The mature lactoferrin folding form prevailed at 150" of the DTT-free incubation.

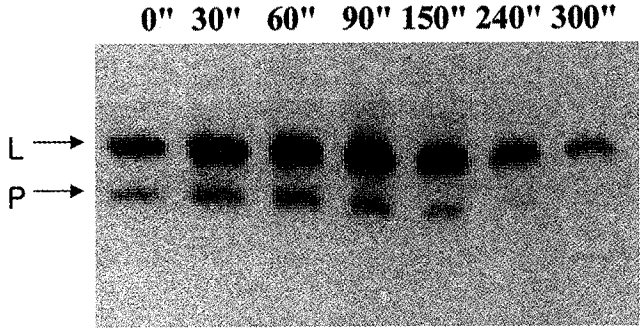

Fig. 4. Interaction between lactoferrin and PDI during the refolding process following DTT unfolding. Structurally matured lactoferrin was subjected to a DTT treatment following an overnight labeling of the cell. As in Figure 3, removing DTT in the incubation media induced the refolding. The lactoferrin (**L**)-PDI (**P**) interaction was assayed by co-precipitation of the two factors by the antibody against lactoferrin. During the refolding process, the PDI-lactoferrin interaction was shown to sustain longer than the original folding process. The interaction is shown to be preserved for up to 4 minutes in this experiment. The interaction appeared to dissociate gradually at 5 minutes with DTT-free incubation.

lactoferrin polypeptides. Partially folded lactoferrin was also found to interact with PDI (Fig. 4). During the refolding process, the PDI-lactoferrin interaction was sustained longer than in the original folding process. The interaction seemed to be preserved for up to 4 minutes in this experiment. However, the interaction appeared to collapse gradually at 5 minutes with the DTT-free incubation. The results showed that PDI is highly reactive with denatured/misfolded peptides to form aggregates, and implies a possible cause from which insoluble protein aggregation emerges. Where PDI served insufficiently during the course of the protein folding, denatured or partially folded peptides might aggregate via intermolecular interaction. Immunoprecipitation data (Fig. 4) indicates that lactoferrin bind extensively to PDI in the course of refolding following DTT-induced unfolding of lactoferrin polypeptides.

Lactoferrin-PDI binding at various incubation temperature Recent studies indicate that a relatively low temperature enhances the proper folding of many mutant proteins, such as thyroglobulins, compared to the temperature of 37°C. To assess the effect of temperature on the PDI activity in lactoferrin folding, fresh mammary gland tissue was labeled overnight. The labeled cells were incubated at three different temperatures: 14, 25, 37°C. The cell was chased for 0, 30, 60 seconds. The extent of PDI-lactoferrin interaction was characterized according to the protein mass, which precipitated along with antisera against lactoferrin. At three time points, the lactoferrin-PDI complex was pulled down by antisera against lactoferrin (Fig. 5). Lactoferrin-PDI binding at 14°C showed a relatively low affinity. At 14°C, PDI showed a relatively low affinity to lactoferrin polypeptides for the three time points. At room temperature (25°C), PDI showed a similar binding affinity to lactoferrin in comparison to 37°C. The PDI binding affinity was quantified according to PDI mass per lactoferrin. The quantitation was made according to the phosphoimaging band quantitation procedure. Following a 30 second incubation at each temperature, PDI was precipitated the most along with the lactoferrin polypeptides. The affinity of lactoferrin-PDI leveled off at 60 seconds. In the case of 25°C, however, the interaction between lactoferrin and PDI appears to be well preserved at each tested time point. The PDI binding to lactoferrin peptide and oligomerization could be affected by temperature. PDI have a chaperoning activity that is required for proper refolding of denatured lactoferrin, which are mediated by the direct interaction with the unfolded polypeptide.

PDI binding to lactoferrin refolding process The lactoferrin folding process can be reversed in the presence of DTT and resumed by removing DTT from the incubation media. Based on this observation, the cell was pulse-labeled for 15 minutes and washed copiously with DTT-free media. Labeled cell lysates were treated with DTT and later DTT was neutralized by IAA. Latoferrin polypeptide began to refold as the DTT concentration diluted out. Lactoferrin was immunoprecipitated by antisera against lactoferrin. Within 30 seconds of refolding, a significant portion of lactoferrin polypeptide appeared at the top of the gel (Fig. 6). This represents an intermolecular aggregation of lactoferrin polypeptides. When incubation continued, the intermolecular aggregation disappeared. This result indicates that molecular chaperones, including PDI, still worked to assist lactoferrin polypeptides folding as the mature form. Indeed, lactoferrin polypeptides occurred as matured monomers.

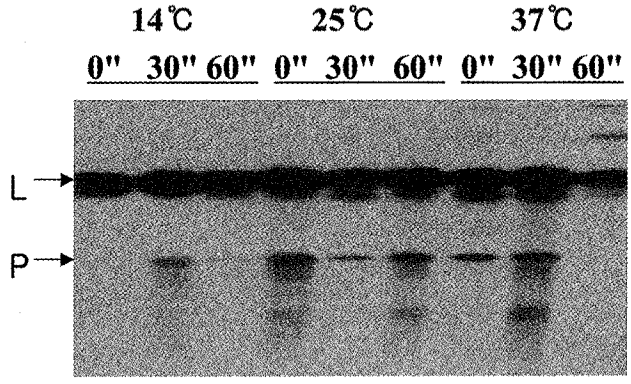

Fig. 5. Temperature effect on the lactoferrin-PDI interaction. The temperature effect on the PDI-lactoferrin interaction was assayed. The incubation temperature was set at 14, 25, 37°C. The cell was chased for 0", 30", 60". The extent of the lactoferrin (**L**)-PDI (**P**) interaction was characterized according to the mass of each protein, which are immunoprecipitated by antisera against lactoferrin. At a 30 second incubation at 14°C, PDI was precipitated the most along with lactoferrin polypeptides. The interaction of lactoferrin-PDI leveled off at 60 seconds. At 25°C, the interaction between lactoferrin and PDI appears to be well preserved at each time point tested. At 37°C, however, minimal PDI-lactoferrin becomes apparent at 60 second even if a strong interaction exists at 0-30".

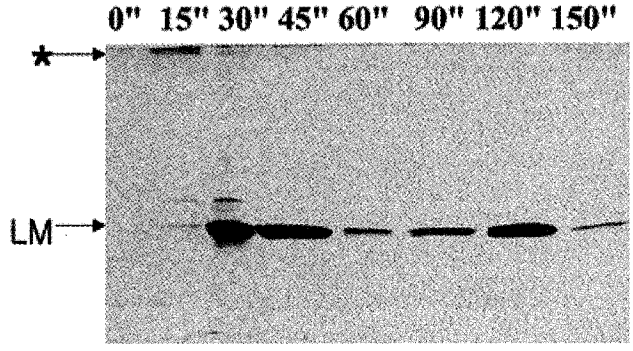

Fig. 6. Refolding of the lactoferrin polypeptide *in vitro*. Labeled cell lysates were treated with DTT then DTT was neutralized by IAA. The latoferrin polypeptide refolded and PDI bound to lactoferrin polypeptides on the refolding process. Lactoferrin was immunoprecipitated by an antisera against PDI. Within 30 seconds of refolding, a significant portion (*) of lactoferrin polypeptide appears at the top of the gel. This represents the intermolecular aggregation of lactoferrin polypeptides. When incubation continued, the intermolecular aggregation disappeared. LM: lactoferrin monomer.

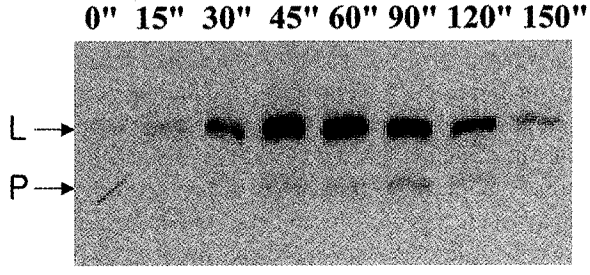

Fig. 7. Lactoferrin-PDI interaction during the protein folding *in vitro*. Cells were labeled overnight and the cell lysate was incubated in the presence of DTT for 5 minutes. DTT was neutralized by IAA for the period indicated. The potential lactoferrin-PDI complex was cross-linked by DSP. The protein complex was immunoprecipitated by antisera against lactoferrin. PDI binding to lactoferrin is considerably extended compared to the *in vivo* counterpart.

The role of PDI was also studied during *in vitro* protein folding of lactoferrin polypeptides. Lysates were prepared from tissue labeled for 5 minutes in the presence of DTT, and the extent of the enhanced lactoferrin folding was assayed. The folding intermediates and lactoferrin aggregates were electrophoresed on a non-denaturing gel. Various folding intermediates were detected during the course of the refolding process when a reducing agent (DTT) was neutralized by IAA (Fig. 6). Under reducing conditions in the ER, denatured lactoferrin polypeptides appeared to aggregate into high molecular weight moieties via an intermolecular interaction. These eventually may precipitate out of solution. During the non-denaturing electrophoresis, these lactoferrin aggregates appeared at the top of the gel. When the IAA neutralizing process continued, lactoferrin polypeptide (unfolded or partially folded) progressed to the fully folded form.

Cells were labeled overnight and cell lysates were incubated in the presence of DTT for 5 minutes. DTT was neutralized by IAA until the time points indicated. A potential lactoferrin-PDI complex was cross-linked by DSST. The protein complex was precipitated by antisera against lactoferrin (Fig. 7). PDI binding to lactoferrin was considerably delayed, but extended. When protein folding of lactoferrin polypeptides (unfolded or partially folded) was even slowed *in vitro* in the presence of DTT, the PDI binding to lactoferrin was increased as *in vivo*. The ratio of PDI per lactoferrin increased, while the mobility of lactoferrin polypeptides remained low; eventually lactoferrin polypeptides disappeared from the gel. This experiment indicates that PDI can play a significant role in protein folding *in vitro*. Consistent with this result from Fig. 6, PDI interacts with lactoferrin polypeptides at a later stage of protein folding. Thus, aggregates and folding intermediates evolve into a fully matured form after PDI binds to the lactoferrin polypeptides, which are on the track of the refolding process. This result also implies that PDI reacts with artificially reduced cysteine residues in a very different manner, slower than the nascent polypeptide's cysteine residue.

Discussion

This study analyzed the peptide folding process of lactoferrin polypeptide. Protein disulfide isomerase (PDI), known to be important in the early stages of protein folding, was the major subject of this experiment. The study was performed under

reducing conditions generated by the addition of DTT. Binding activity of PDI to lactoferrin polypeptides was characterized by the co-immunoprecipitation of the two proteins. A non-denaturing PAGE analyzed Lactoferrin folding intermediates via the difference in the mobility. In addition, the folding intermediates were characterized in terms of binding with PDI under various environments, which are believed to affect the binding. The environments refer to reducing or oxidizing conditions, as well as a variety of temperatures.

The results indicate that lactoferrin folding may require an excessive binding of PDI, and its binding is relatively brief compared to other typical nascent polypeptides. Based upon these results, PDI is believed to play an important role in nascent lactoferrin folding. The amount of lactoferrin interacting with PDI increases up to half a minute and sharply decreases beyond that time point. This result confirms that lactoferrin polypeptides heavily interact with PDI for relatively short lengths of time at the early stage of peptide folding. At this stage, the presence of DTT still retarded the refolding of lactoferrin polypeptides and the majority of lactoferrin polypeptides were found to be folding intermediates.

The interaction between lactoferrin and PDI appeared to be better when kept at a lower temperature (25°C). PDI binding to lactoferrin peptide and oligomerization could be augmented by a lower temperature. There appears to be a greater requirement for PDI chaperone activity during the refolding process. Also, PDI may have a chaperoning activity that is required for the proper refolding of denatured lactoferrin, which is mediated by the direct interaction with the unfolded polypeptide. Therefore, these results suggest that PDI provides an additional chaperoning activity in the protein refolding process.

According to *in vitro* experiment data, molecular chaperones, including PDI, were still functional in assisting the lactoferrin folding into the mature form. Consistent with this result, PDI interacts with lactoferrin polypeptides for an extended period of time until the lactoferrin polypeptide achieved a decent folding structure *in vitro*. Intermolecular aggregates and folding intermediates matured into a functional form after PDI binding to the lactoferrin polypeptides, which are on the track of the refolding process. This result also implies that PDI reacts with artificially reduced cysteine residue in a very different manner, slower than the nascent polypeptide's cysteine residue.

Elucidation of the mechanism of the formation of insoluble aggregates, and the intracellular digestion of proteins, is essential to resolve the problems in the limited overexpression of useful proteins (Lee, 1998; Kajino *et al.*, 2000; Sideraki and Gilbert, 2000). In this study the role of PDI on the aggregation and digestion of denatured proteins were investigated. Denatured lactoferrin was shown to heavily bind with PDI, and aggregate among the polypeptides; thus, causing an instant multi-molecular aggregation among denatured polypeptides. Partially folded lactoferrin was also found to interact with PDI. The results show that PDI is highly reactive with denatured/misfolded peptides to form aggregates and to initiate digestion. It also suggests a possible mechanism by which insoluble protein aggregation can proceed. Because the understanding of the quality control system for which PDI is responsible is very important, we expect it to be profitable (Rowling and Freedman, 1993). Among others, it will affect the development of genetic engineering. When genetically engineered lactoferrin is expressed in a heterogeneous system, the quality control system destroys lactoferrin, or forms an inclusion body with very low solubility. This phenomenon makes it difficult to separate useful lactoferrin, and presents the whole field of biological engineering with a dilemma. It is expected that an understanding of the lactoferrin quality control system will contribute to an increase in lactoferrin production efficiency.

Taken together, this study suggests that PDI has a chaperoning activity required for proper refolding of denatured lactoferrin, which is mediated by direct interaction with the unfolded polypeptide. This result suggests that an interaction with the mature portion of the protein also is important for the role of PDI. The PDI-lactoferrin interaction levels increase at 25°C. In addition, the participation of PDI in protein refolding is prolonged at a lower temperature (25°C). Therefore, these results suggest that PDI provides an extended chaperoning activity in refolding processes; however, there appears to be a greater requirement for the PDI chaperone activity in the refolding of lactoferrin polypeptides.

Acknowledgments A Korea Research Foundation Grant (KRF-1998) supported this work. Part of this work was supported by the Brain Korea 21 Project.

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