Protein Disulfide Isomerase and Newly Synthesized Procollagen Chains form Higher-order Structures in the Lumen of the Endoplasmic Reticulum*

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A number of proteins that act as necessary catalysts for correct protein folding and oligomerization in the endoplasmic reticulum (ER) are known to be retained in the organelle via the KDEL-receptor mediated retrieval mechanism. However, a complementary system that may help to retain these proteins in the organelle lumen has been suggested to exist and likely involves physical protein-protein interactions at the level of endoplasmic reticulum (ER) itself. In this report, we provide both morphological and biochemical evidence in support of this proposal. We show that in collagen-secreting human skin fibroblasts, protein disulfide isomerase and newly synthesized procollagen chains exist predominantly in an “aggregated” state, and form a reticular-like matrix in the ER lumen in vivo. The size of the aggregates was found to be variable, and may exceed 1.5 million Da. Aggregate formation appeared to be transient and to involve multiple types of protein-protein interactions, including formation of aberrant disulfide bonds. Association of protein disulfide isomerase, on the other hand, was found to require at least partly function-related disulfide bonds. These results support the existence of a reticular-like matrix in the ER lumen, and suggest that aggregation may be part of the normal maturation pathway during collagen biosynthesis.

Through a series of experiments, it has been well demonstrated that the retention of a number of luminal protein folding catalysts, such as binding protein (BiP),1 GRP94/endoplasmin, and protein disulfide isomerase (PDI) is based on the KDEL-receptor-mediated retrieval mechanism (1–3). There is circumstantial evidence, however, to suggest the existence of other, perhaps complementary systems that may help to retain these proteins in the ER lumen. For example, previous mutational analyses have shown that the KDEL-deletion mutants of BiP and PDI (4, 5) or calreticulin (6) are secreted from the transfected cells at a much slower rate than expected if the KDEL-sequence was the only signal dictating retrieval in the ER. Similarly, deletion of the double lysine retrieval motif (KXX/KXX) from an endogenous transmembrane enzyme, glucuronosyltransferase, does not result in the loss of ER retention (7). In addition, endogenous proteins in the ER lumen, in contrast to KDEL-tagged reporter molecules, do not seem to receive any Golgi-specific oligosaccharide modifications (8, 9), suggesting that they do not recycle to a great extent between the ER and the (pre)Golgi compartment where the KDEL-receptor has been shown to reside (10).

One possibility that could restrict continuous recycling of the KDEL-containing proteins between the first two secretary compartments, likely involves physical protein-protein interactions at the level of ER. In fact, it has been extensively hypothesized that luminal proteins may even form a matrix or a gel in the ER lumen (3, 11, 12). Direct evidence for the existence of such a putative reticular matrix in the ER lumen has been hard to find, but this possibility was highlighted when it was found that calcium ionophores were able to induce secretion of PDI, BiP, and GRP94/endoplasmin from murine fibroblasts (13).

In this report, we have analyzed the physical nature of the ER lumen by using PDI, and its main substrates in human skin fibroblasts, i.e. various procollagen folding and assembly intermediates as markers. Given that both proteins were found in association with higher-order structures in the ER lumen, our data strongly argue against the freely soluble nature of both proteins under in vivo conditions. Rather, the results support the existence of a reticular-like matrix in the ER lumen in vivo that in collagen-secreting human skin fibroblasts appears to be made up predominantly of newly synthesized procollagen chains and associated protein folding catalysts such as PDI. In contrast to murine fibroblasts (13), matrix assembly in human cells appeared to require multiple kinds of protein-protein interactions.

EXPERIMENTAL PROCEDURES

Chemicals—A monoclonal antibody used for the identification of PDI has been prepared against the purified protein as described previously (14). It was used at a concentration of 0.5–10 μg/ml. Affinity-purified rabbit antibodies against human procollagen type I and type III α-chain N-terminal propeptides were from Dr. Leila Risteli (University of Oulu, Oulu, Finland). Tetramethylrhodamine isothiocyanate-conjugated swine anti-rabbit (Bio-Rad), or peroxidase-conjugated sheep anti-rabbit and anti-mouse Fab-fragments (BioSys, Compiegne, France) were used as secondary antibodies for immunoblotting and immunoelectron microscopy with their corresponding substrates (the ECL detection method, Amersham, Bucks, United Kingdom; diaminobenzidine, Sigma). Calcium ionophores, A23187 and thapsigargin, were obtained from Calbiochem. Stock solutions (10 mM and 50 μM, respectively) were dissolved in Me2SO.

Cell Culture and Drug Treatments—Human embryonic skin fibroblasts were grown in minimal essential medium supplemented with Eagle’s salts, glutamine, nonessential amino acids, 10% newborn calf serum, penicillin-streptomycin (Life Technologies, Inc.). Typically, the

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‡ The abbreviations used are: BiP, binding protein; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.
culture medium was supplemented with 50 μM ascorbic acid. Alkylation of cells with 20 mM N-ethylmaleimide was done as described earlier (15). Treatment of the cells with calcium ionophores (A23187, 10 μM; thapsigargin, 50 μM) were performed in the absence of serum.

Immunohistochemistry—Cells were processed for indirect immunofluorescence as described elsewhere (16). All incubations, unless otherwise stated, were carried out in the presence of 0.05% saponin. Stained specimens were examined using an epifluorescence microscope (Leitz, Aristoplan) equipped with appropriate filters. Electron microscopy was performed as described earlier (16, 17). Briefly, cells grown on Petri dishes either with or without calcium ionophores were washed with PBS, and fixed immediately with 3.7% formaldehyde, 0.1% glutaraldehyde mixture (in PBS) for 60 min at 20°C. Free aldehyde groups were quenched with 50 mM ammonium chloride (in PBS for 15 min), and then stained with the primary antibodies (anti-PDI and anti-procollagen propeptide antibodies) followed by peroxidase-conjugated secondary antibodies. All antibody incubations were done in the presence of 0.1% saponin and 0.1% bovine serum albumin. After post-fixing with 2.5% glutaraldehyde, diaminobenzidine (0.5 mg/ml) was added and peroxidase reaction was allowed to proceed 15–30 min on ice, after which the cells were Epon-embedded and sectioned using conventional procedures.

Immunogold-labeling on thin cryosections was carried out as described in FrifINO et al. (18). Stained specimens were examined using the Philips 410 LX transmission electron microscope with an acceleration voltage of 60 kV. Quantitation of the surface area in the micrographs was carried out by using a computer-assisted image analysis program (MCID-M1, Imaging research, St. Catharines, Canada).

Size-exclusion Chromatography—Cells (at about 80% confluency) growing on a 60-mm diameter Petri dishes were washed twice with PBS, scraped with a rubber policeman on ice either with or without prior alkalization, and collected by centrifugation (300 × g, 10 min, 4°C). Pelleted cells were dissolved by using the Triton X-114 phase separation method (19). Briefly, 1 ml of ice-cold 2% Triton X-114 solution (in TKM buffer: 50 mM Tris (pH 7.4), 10 mM KCl, 1 mM MgCl₂) supplemented with the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin were added to the cell pellet for 30 min on ice. The aqueous phase containing >95% of total PDI (17) was applied directly to the Bio-Gel 1.5 m size exclusion chromatography column equilibrated with the TKM buffer. In some cases, frozen cells were used as a starting material for the experiments. The size fractionated samples were then trichloroacetic acid-precipitated (10% trichloroacetic acid, 1 h on ice) and analyzed by SDS-PAGE (20) and immunoblotting with the monoclonal anti-PDI antibody and the anti-procollagen propeptide antibodies using the ECL detection system (Amersham). Computer-assisted image analysis program was used to quantify the content of the stained bands on x-ray films.

Extraction of PDI and Procollagen with Saponin—Similarly to a previous study (21), extraction of PDI and procollagen chains from intact or alkylated cells with saponin were performed after washing plates twice with PBS by using 50 mM Tris, 250 mM sucrose (isosmotic) buffer (pH 7.4). Extraction was done on ice for 30 min by adding slowly 1 ml of ice-cold buffer supplemented with freshly made 0.1% saponin (10% stock solution in H₂O), 150 mM KCl, and/or 100 mM DTT. The extraction solution was collected and concentrated by using a 300-kDa filter. After SDS-PAGE and immunoblotting with the anti-PDI and anti-procollagen propeptide antibodies, the stained bands on the blots were visualized using the ECL detection system and an x-ray film (Fuji RX). Quantitation of the bands were performed by using the MCID-M1 image analysis program.

Immunoprecipitation Experiments—Immunoprecipitation analyses (16) were carried out after pulse-labeling cells for 10 min to 2 h with [³⁵S]methionine (0.2 μCi/ml; Amersham). Cells were solubilized in 1 ml of ice-cold RIPA buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, 5 mM EDTA, 150 mM NaCl, pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride to inhibit protease activity. The same buffer was used throughout the whole immunoprecipitation protocol including washings. Immunocomplexes (containing rabbit antibodies) were collected with protein A-agarose beads (Sigma) before analysis by SDS-PAGE (20) either under reducing or non-reducing conditions. Immunoprecipitated bands in dried gels were visualized using an x-ray film.

RESULTS

PDI Is Unevenly Distributed within the ER Lumen—The anti-PDI monoclonal antibody used in this study has been previously demonstrated to recognize from the total cell lysates of human fibroblasts only one major protein with the expected relative mobility for PDI (57 kDa) on SDS-PAGE (14, 17). The antibody is thus highly specific for PDI, and does not cross-react with other KDEL-containing proteins such as BiP or GRP94/endoplasmic. In indirect immunofluorescent experiments, the antibody stains specifically the reticular ER, including the nuclear envelope (Fig. 1A). Immunoperoxidase staining at the electron microscopical level (Fig. 1B) confirmed that PDI is distributed throughout the ER lumen and the nuclear envelope, but appears to be excluded from other organelles such as mitochondria (Fig. 1B) and the Golgi complex (Fig. 1C). The staining pattern within the ER lumen was not uniformly diffuse, as would be expected if PDI was a freely soluble protein in the ER lumen. Rather, the staining pattern was granular within the ER lumen (Fig. 1, C and D). These PDI-positive granules were quite uniform in size, having an average diameter of 40–50 nm (Fig. 1, C and D).

Immunogold staining gave comparable results (Fig. 2). The gold particles showed a tendency to congregate into small groups that often located above some electron-dense structures seen in the micrographs. Quantitation of the gold particles into those that were less or more than 50 nm apart from one another revealed that out of 1086 particles totally examined, 678 particles (62.5%) belonged to the first group, while 408 particles (37.5%) existed more than 50 nm apart from its neighboring particle. Assuming that the 207 gold particles shown in Fig. 2 were spread evenly within the measured surface area (4.3 μm²) of the ER lumen, the average distance between the gold particles would be 162 nm. Thus, the observed distribution of gold particles is in good agreement with the immunoperoxidase staining data. These findings gave the first indication against the freely soluble state of PDI in vivo.

PDI Association Involves Multiple Kind of Protein-Protein Interactions—To provide biochemical evidence for the association of PDI with the luminal constituents of the ER, we first solubilized cells with Triton X-114 (19) and subjected solubilized proteins onto a size-exclusion chromatography column (Bio-Gel 1.5 m; Fig. 3). Previously (17), we have shown that more than 95% of total cellular PDI can be recovered from the aqueous phase (TKM buffer: 50 mM Tris buffer, pH 7.4, 10 mM KCl, 1 mM MgCl₂) using this method. SDS-PAGE and immunoblotting analyses of the size-separated fractions with the anti-PDI monoclonal antibody revealed that PDI eluted from the column as three wide peaks (Fig. 3a) with a much higher molecular size (>0.6–1.5 × 10⁶ Da) than expected from the size of PDI monomer (57 kDa) or its dimeric form (about 120 kDa). The 300-kDa species, which comprised about half of the total PDI in the sample, and was the major form detected when frozen cells (Fig. 3a) were used as a starting material, most probably corresponds to the detergent-solubilized prolyl-4-hydroxylase enzyme. This enzyme is a heterotetrameric protein (a₂b₂, 250 kDa) the β-subunit of which is identical to PDI (22, 23). An identical elution profile for PDI was obtained when cells were alkylated with 20 mM N-ethylmaleimide (15) prior to solubilization (data not shown), indicating that the high molecular size PDI-protein complexes do not arise because of possible artificial cross-linking during solubilization.

Our permeabilization experiments with 0.1% saponin (21) provided additional evidence against the freely soluble state of PDI in vivo. This was evidenced by our immunoblotting experiments, which showed that only a minor portion (<20%) of the
total PDI could be extracted from intact (Fig. 3b) or alkylated cells (data not shown) with saponin using low salt-containing buffers such as 50 mM Tris, 250 mM sucrose (pH 7.4), or TKM. This was confirmed by indirect immunofluorescent staining with the monoclonal anti-PDI antibody which demonstrated the presence of PDI within the ER of the treated cells at comparable amounts to untreated cells (data not shown). However, addition of salt (150 mM KCl) into the extraction buffer (0.1% saponin/Tris/sucrose buffer) increased extraction efficiency from 17 to 55% in the case of PDI. The most efficient
extraction (about 80% of total PDI) was obtained by including 100 mM dithiothreitol (DTT) into the above salt-containing extraction buffer. Thus, both electrostatic and disulfide-mediated protein-protein interactions appear to be responsible for the poor extractability of PDI from intact cells under the conditions used.

In contrast to murine fibroblasts (13), PDI association was found to be a rather calcium-independent phenomenon. This

**FIG. 2.** Immunogold staining of human fibroblasts with the anti-PDI monoclonal antibody. Note the localization of the gold particles above electron dense structures within the ER lumen (a) and their predominant congregation close to each other (b, the gold particles are shown alone). Bar, 100 nm.
was demonstrated first by showing that addition of either calcium (3 mM) or calcium chelators (EDTA, 5 mM) into the extraction buffer, did not affect the extraction efficiency of PDI from the cells (data not shown). Second, we found that calcium ionophores, A23187 (10 μM) and thapsigargin (50 nM), induced secretion of only a minor portion of PDI (<10%) from the cells during the 6-h incubation period (data not shown). Third, morphologically similar 50-nm PDI-containing granules to those observed in untreated cells (Fig. 1) were detected within the ER lumen and the nuclear envelope, despite the use of the drugs and the severe dilatation of the ER cisternae caused by these drugs (Fig. 4). Because of dilatation, PDI-containing structures could be detected both in the luminal space and in close association with the ER membranes (Fig. 4b). Together, these structures appeared to form a reticular-like matrix within the ER lumen.

Previous structural analyses have shown that PDI is active as a homodimer and in each of its subunits there are duplicated domains that show strong homology to thioredoxin active site domains (24, 25). Both of the cysteine residues in these active site domains (-CGHC-, single amino acid code) appear to be highly reactive and to be involved in dithiol-disulfide interchange reactions. Alkylation of any of these active site cysteines is known to freeze the existing disulfide bonds, possibly by preventing further disulfide interchange reactions (26). To test whether PDI interaction involved such function-related disulfide bonds, we alkylated cells prior to solubilization and analysis by SDS-PAGE and immunoblotting with the anti-PDI antibody. In addition to PDI monomer (57 kDa; Fig. 5) we found three to four distinct bands which had much higher molecular size (145–170 kDa on SDS-PAGE) than the PDI monomer (Fig. 5, lane 1). These bands were absent either in non-alkylated samples (lane 2), or if the samples (alkylated or not) were reduced with mercaptoethanol before SDS-PAGE (lanes 3 and 4). It was concluded that association of PDI at least with some luminal constituents involves function-related disulfide bonds.

Incompletely Folded Procollagen Chains Form Disulfide-mediated Aggregates in the ER Lumen—Human skin fibroblasts are known to secrete mainly type I and III procollagen chains both in vivo and in vitro (27). The rate-limiting step for the secretion of procollagen is the formation of procollagen triple helices in the ER. Trimerization starts from the C-terminal ends of the procollagen chains and requires correct positioning of several intra- and interchain disulfide bonds within the procollagen triple helix. PDI is known to catalyze these dithiol-disulfide interchange reactions (28).

To analyze whether incompletely folded procollagen chains also form higher-order structures in the ER lumen, we first used immunoelectronic microscopy after staining A23187-treated cells with antibodies directed against the N-terminal propeptide regions of both type I and III procollagen chains. As the epitope-containing propeptide regions are cleaved off after transport of fully folded trimerized procollagen chains to the extracellular space (28), either antibody should recognize only intracellular procollagen monomers and their various folding or assembly intermediates. Our immunofluorescent experiments showed that both antibodies do stain preferentially the ER lumen (data not shown). Subsequent immunoperoxidase staining at the EM level (Fig. 6a) revealed that the anti-propeptide antibodies decorated morphologically similar structures, i.e. a reticular-like matrix in the ER lumen (compare with Fig. 4), suggesting that newly synthesized procollagen chains also associate with or are part of the putative matrix in the ER lumen in vivo.

Similarly to PDI, efficient extraction of procollagen chains (type III) with 0.1% saponin could not be achieved with low salt-containing buffers (Fig. 6b). Addition of salt (150 mM KCl) to the extraction buffer increased the amount of solubilized procollagen from 8% (control) to about 30% of the total amount of the protein detected in the cells. As with PDI, most efficient extraction was obtained by including both salt and 100 mM DTT in the extraction buffer. This resulted in almost quantitative release (>90%) of type III procollagen chains from the cells (Fig. 6b). These findings together with the observation that Triton X-114 solubilized type III procollagen chains elute from the size-exclusion chromatography column exclusively at the void volume (data not shown) suggested that newly synthesized procollagen chains exist predominantly as high molecular size, disulfide-bonded aggregates in the ER lumen in vivo.

2 In this report, the word “aggregate” is used in a broad sense and does not exclude, e.g. the existence of proteins as large protein complexes.
We confirmed this by using both immunoblotting (data not shown) and immunoprecipitation (Fig. 7) approaches. The data obtained with the anti-procollagen propeptide antibodies demonstrated that a substantial portion of type I and type III procollagen chains form high molecular size aggregates that did not enter the separating gel under non-reducing conditions (Fig. 7a, lanes 1 and 2). These procollagen-containing aggregates were detected after pulse-labeling of the cells for only 10 min (data not shown). The aggregates were no longer present if the samples were reduced before SDS-PAGE (lanes 3 and 4). Reduction resulted in a concomitant appearance of only 150–170 kDa protein bands that represent procollagen \((\alpha)_1\) and \((\alpha)_2\) chain monomers. Thus, aggregation of both type I and type III procollagen chains shortly after their synthesis predominantly involves formation of aberrant disulfide bonds.

A number of experiments were designated to verify that the procollagen bands seen on top of the separating gel represent aggregated procollagen chains, and not, e.g. completely folded procollagen chains (expected molecular size is about 450 kDa) ready to be secreted into the extracellular space. First, we cultured cells for 2 days in ascorbic acid-depleted media to inhibit trimerization of procollagen chains, and pulse labeled cells for 2 h with \([35S]\)methionine before solubilization and immunoprecipitation. Depletion of ascorbic acid, however, did not cause the disappearance of the bands at the top of the separating gel, as was expected to happen if the bands represented fully folded procollagen chains (Fig. 7b, lane 2). Similar results were also obtained when \(\alpha\),\(\alpha\)-dipyridyl was used to inhibit trimerization of procollagen chains (data not shown).

The aggregates were also susceptible to proteolytic attack by trypsin (Fig. 7, lane 4), in contrast to completely folded procollagen chains. These results thus exclude the possibility that the high molecular size type I and III procollagen bands represent merely fully matured procollagen chains.

**DISCUSSION**

In the present study, we have analyzed the physical nature of the two major proteins found in the ER lumen in collagen-secreting human skin fibroblasts, namely PDI which is an ER-resident enzyme required for correct protein folding and

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**Fig. 4.** Immunoperoxidase staining with a monoclonal anti-PDI antibody of the cells treated with A23187. a, note the dilation of the nuclear envelope in the drug-treated human fibroblasts, and the peroxidase-stained reticular-like matrix (closed arrow in a) within the ER lumen. Open arrows (a) denote to unaffected ER cisternae in the same cell. b, the electron micrograph shows stained PDI-containing structures within the ER lumen at a higher magnification. Note that in addition to the luminal space, stained structures were detected in close association with the ER membrane (arrowheads). Small arrows denote to the double-layered nuclear envelope. n, nucleus; er, endoplasmic reticulum. Bars, 100 nm.

**Fig. 5.** Immunoblotting of total cell lysates with the anti-PDI monoclonal antibody. Cells either with or without prior alkylation were solubilized directly with SDS sample buffer. Half of the samples were reduced and subjected to SDS-PAGE. Immunoblotting of the samples with the anti-PDI monoclonal antibody shows three PDI-containing protein bands in the 145–170-kDa range (lane 1), in addition to PDI monomer (57-kDa band). These high molecular size bands (lane 1) were detected only in alkylated samples under non-reducing conditions (compare with lanes 2–4). NEM, N-ethylmaleimide; ME, mercaptoethanol.
oligomerization, and type I and III procollagen chains which are secretory proteins and appear to be the main substrates for PDI in these cells. Our biochemical data indicated that both proteins associate with higher-order structures or form large molecular size protein complexes or aggregates in the ER lumen in vivo. The findings are thus in good agreement with previous biochemical studies in which PDI and procollagen have been shown to co-immunoprecipitate with BiP, GRP94 (13), and Hsp47 (29). Calnexin is also found in association with incompletely folded glycoproteins in the ER lumen (30, 31) and PDI associates with the α-subunit of prolyl-4-hydroxylase via a non-disulfide-mediated fashion (32). Collectively, these findings are compatible with the morphological data reported here, strongly supporting the existence of a putative reticular-like matrix in the ER lumen.

In contrast to murine fibroblasts, multiple types of protein-protein interactions appeared to be responsible for the observed protein-protein interactions. Our extraction experiments on intact cells with saponin indicated that PDI associates with its counterparts via both electrostatic and disulfide-mediated interactions (Fig. 3b). In the case of PDI, at least part of the latter represented function-related disulfide bonds, i.e. those that can be detected only by the use of alkylating agents. Association probably also involves hydrophobic interactions, although these remain undetected in our experiments because of the need to use detergents for solubilization of cells. Similarly to PDI, efficient extraction of newly synthesized procollagen chains with saponin required the use of a reducing agent in the presence of salt. This finding together with our immunoprecipitation experiments demonstrated that procollagen aggregation is mediated predominantly by the formation of aberrant disulfide bonds. The redox state of the ER lumen is known to favor disulfide bond formation in vivo (33). These multiple types of protein-protein interactions probably explain our inability3 to cause any marked secretion of PDI or BiP from the cells with any of the drugs previously used for similar studies (calcium ionophores, 2-mercaptoethanol, DTT, pH-perturbing agents; see Refs. 13, 29, and 34–36).

Aggregation is a general feature during protein synthesis (15, 37–39). Most often, aggregation is considered as part of the non-productive, dead-end pathway for folding which eventually leads to degradation via a non-lysosomal mechanism. Previous studies have shown, however, that aggregation does not necessarily lead to degradation. For example, aggregation appears to be responsible for the formation of intracellular granules (40) or Russel bodies (36) and in these cases appears to serve as a specific sorting event by which specific proteins can be sorted out from other proteins in the ER lumen. Aggregation may also be part of the normal maturation pathway during protein synthesis. Detailed kinetic analyses (41) have demonstrated thyroglobulin aggregates early during thyroglobulin biosynthesis,

\(^3\) S. Kellokumpu, M. Suokas, L. Risteli, and R. Myllylä, unpublished data.
and that these aggregates are transient and precede the appearance of fully matured polypeptide chains.

Correct disulfide bond formation rarely occurs de novo. Rather, it requires reshuffling of existing intra- and/or intermolecular disulfide bonds (24, 25, 42, 43). Our findings that newly synthesized procollagen chains also form disulfide-mediated aggregates early during their synthesis (10-min pulse, data not shown) and that these aggregates disappear during the subsequent 2-h chase period (data not shown) suggest that procollagen biosynthesis may also involve transient aggregation in the ER lumen. This view is supported by the observation that PDI association was mediated at least partly by function-related disulfide bonds (Fig. 4). Previous studies have provided evidence that PDI is capable of solubilizing aggregated proteins (25, 43) or to unfold kinetically trapped folding intermediates (44). Other protein folding catalysts also have been shown to possess such “disaggregase” activity, as bacterial dnaK in combination with dnaJ (45) and yeast HSP104 (46) can rescue heat-denatured luciferase-reporter enzyme under in vivo conditions. Diassembly of clathrin cages or activation of signaling events mediated by protein kinases or steroid receptors also appears to involve members of the Hsp70 and Hsp90 protein family which recognize mature proteins and facilitate their unfolding or disassembly (for a review, see Ref. 47). Thus, the ability of the luminal folding catalysts to assist correct protein folding may partly relate to their ability to dissolve aggregated proteins in the ER lumen in vivo.

Aggregate formation in the ER lumen would offer several benefits to the cell. For example, it would allow high concentrations of both the folding catalysts and their substrates to be kept in close proximity to each other, thereby increasing the probability of correct folding and oligomerization processes. In addition, aggregate formation itself would efficiently restrict premature export of incompletely folded proteins out of the organelle, and thus their unnecessary recycling between the ER and the Golgi compartment. Evidence that disulfide-mediated protein-protein interactions in the ER lumen can indeed be responsible for the retention of proteins has been recently reported (48). As an additional benefit, the export of the associated molecular chaperones and PDI would also be simultaneously minimized, and may thus provide an explanation for the slow secretion observed with the KDEL-deletion mutants of BiP (4), PDI (5), and calreticulin (6) in transfected cells. On the other hand, the suggested aggregate dissolution by PDI and by other molecular chaperones as well, would provide a simple means to release only correctly folded proteins from the aggregates and thus to segregate incompletely folded proteins from the folded (i.e. transport competent) ones solely on the basis of their different solubility.

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