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Interchain Disulfide Bonds Promote Protein Cross-Linking during Protein Folding[1]

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We provide evidence that *in vitro* protein cross-linking can be accomplished in three concerted steps: (i) a change in protein conformation; (ii) formation of interchain disulfide bonds; and (iii) formation of interchain isopeptide cross-links. Oxidative refolding and thermal unfolding of ribonuclease A, lysozyme, and protein disulfide isomerase led to the formation of cross-linked dimers/oligomers as revealed by SDS–polyacrylamide gel electrophoresis. Chemical modification of free amino groups in these proteins or unfolding at pH < 7.0 resulted in a loss of interchain isopeptide cross-linking without affecting interchain disulfide bond cross-linking. Furthermore, preformed interchain disulfide bonds were pivotal for promoting subsequent interchain isopeptide cross-links; no dimers/oligomers were detected when the refolding and unfolding solution contained the reducing agent dithiothreitol. Similarly, the Cys326Ser point mutation in protein disulfide isomerase abrogated its ability to cross-link into homodimers. Heterogeneous proteins become cross-linked following the formation of heteromolecular interchain disulfide bonds during thermal unfolding of a mixture of of ribonuclease A and lysozyme. The absence of glutathione and glutathione disulfide during the unfolding process attenuated both the interchain disulfide bond cross-links and interchain isopeptide cross-links. No dimers/oligomers were detected when the thermal unfolding temperature was lower than the midpoint of thermal denaturation temperature.

Key words: disulfide bonds, protein cross-linking, protein disulfideisomerase, oxidative refolding, thermal unfolding.

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Protein cross-linking has been implicated in several biologic processes, including the stabilization of fibrin clotting during blood coagulation (*1*), synthesis of apoptotic bodies in cells undergoing apoptosis (*2*), terminal differentiation of epidermal cells (*3*), formation of hair and nails (*4*), and formation of cataracts (*5*). Interchain isopeptide bonds between a free epsilon amino group of a lysine residue on one polypeptide chain and a gamma carboxyl group of a glutamine residue on another polypeptide chain, are considered to be involved in these processes. Similarly, cross-linking/oligomerization of proteins is common in a group of diseases characterized by abnormal protein deposition, including Huntington's disease, Alzheimer's disease, Parkinson's disease, and prion disease. However, the molecular mechanisms for converting monomeric/functional forms of proteins into cross-linked oligomeric forms are not well understood. Two notions have been proposed. One group concurs that conformational changes, including partial changes in protein secondary structure, are responsible for protein cross-linking and oligomerization (*6*, *7*); whereas another group believes that enzyme-mediated isopeptide bond cross-links catalyzed by transglutaminase (*8*, *9*) contribute to the oligomerization.

Disulfide bonds formed as a result of oxidation of cysteine residues play an important role in a protein's conformational stability and function (*10*, *11*). Similarly, these bonds are considered crucial in the intermediate folding state of a protein (*12*). It has been observed recently that disulfide bonds mediate adhesion of platelets in blood coagulation (*13*) and retina cell adhesion during retina development (*14*). In this paper, we demonstrate that oxidative refolding and thermal unfolding of ribonuclease A, lysozyme, and protein disulfide isomerase (PDI) lead to interchain cross-links, which is preceded by the formation of interchain disulfide bonds.

EXPERIMENTAL PROCEDURES

Refolding of Ribonuclease A—Reduced, denatured bovine ribonuclease A (Sigma Chemical, St. Louis, MO) at a concentration of 0.1 mM was incubated at 37°C for 3 h in Tris-HCl buffer (25 mM, pH 7.6) containing EDTA (1.0 mM), GSH (2.0 mM), and GSSG (1.0 mM). The preparation of reduced, denatured ribonuclease A was carried out as described previously (*15*).

Trinitrophenylation of Ribonuclease A—Native bovine ribonuclease A (10 mg) was dissolved in 1 ml of 200 mM Tris-HCl buffer (pH 8.5) and incubated at 37°C for 4 h with 0.05 ml of 5% 2-,4-,6-trinitrobenzenesulfonin acid. The mixtures were then extensively dialyzed against 25 mM Tris-HCl (pH 7.6).

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Abbreviation: PDI, protein disulfide isomerase.

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Thermal Unfolding—Native bovine ribonuclease A, chicken lysozyme (Sigma), or a mixture of the two proteins, each at a concentration of 0.1 mM, was incubated for 30 min in 25 mM Tris-HCl (pH 7.6) containing EDTA (1.0 mM), GSH (2.0 mM), and GSSG (1.0 mM). The incubation temperatures were selected according to the midpoint temperature of the thermal denaturation. Ribonuclease A was incubated at 65°C, lysozyme at 75°C, and the mixture of ribonuclease A and lysozyme at 75°C. For PDI, 0.005 mM recombinant proteins were incubated at 65°C for 30 min in 25 mM Tris-HCl (pH 7.6) containing GSH (2.0 mM), GSSG (1.0 mM), and calcium chloride (10 mM).

Construction and Purification of Recombinant Human PDI—Site-directed mutants were made on pBluescript-PDI plasmids (*16*), which contained a 1.5-kb, wild-type human PDI cDNA without the signal peptide sequence. The mutants were generated by using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The synthetic oligonucleotides used were as follows: 5′-CTGAAGAAGGAAGAGAGCCCGGCCGTGCGCCTC-3′ (Cys295-Ser, sense primer), 5′-GAGGCGCACGGCCGGGCTCTCTTCCTTCTTCAG-3′ (Cys295Ser, anti-sense primer), 5′-GGATCACAGAGTTCAGCCACCGCTTCCTGGAG-3′ (Cys326-Ser, sense primer), and 5′ CTCCAGGAAGCGGTGGCTGAACTCTGT GATCC-3′ (Cys326Ser, anti-sense primer). The three mutants Cys295Ser, Cys326Ser, and Cys295/326Ser were confirmed by DNA sequencing. The 1.5-kb cDNAs cut from corresponding pBluescript-PDI plasmids were subcloned into separate pTrcHisC expression plasmids (Invitrogen, Carlsbad, CA). *Escherichia coli* (BL21) cells harboring the expression plasmids were grown to an optical density of 0.6 at 600 nm. Recombinant protein expression was induced by 0.4 mM IPTG for 4 h. The cells were harvested by centrifugation and resuspended by vortexing in lysis buffer (50 mM Tris-HCl, pH 8.5, 100 mM NaCl, and 0.10 mg/ml lysozyme). The lysates were incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were then transferred to a clean tube. The recombinant PDIs were purified by using Talon metal affinity resin (Clontech, Palo Alto, CA) under nondenaturing conditions, according to the manufacturer's protocol.

SDS-PAGE and Western Blot Analysis—SDS-PAGE and Western blotting were performed as described (*17*). For Western blotting, membranes containing transferred proteins were probed with a 1:2,000 dilution of anti-PDI polyclonal antibody (StressGen Biotech, Collegeville, PA), and the bound antibody was detected using a 1:5,000 dilution of anti–rabbit horseradish peroxidase–conjugated antibody and the ECL detection reagents (Amersham Pharmacia Biotech, England).

RESULTS AND DISCUSSION

Interchain Disulfide Bonds Lead to Protein Cross-Linking—Reduced, denatured ribonuclease A was incubated in a glutathione redox buffer for oxidative refolding, and the reactants were analyzed by SDS-PAGE. As demonstrated in Fig. 1, refolding was accompanied by cross-linking of ribonuclease A molecules. Thus, the cross-linked dimers/oligomers of ribonuclease A were observed in SDS-PAGE (Fig. 1, lane 2). Derivatization of free amino groups with trinitrobenzenesulfonin resulted in a complete loss of ribonuclease A dimer/oligomer formation (Fig. 1, lane 3). However, dimers/oligomers of trinitrophenylated ribonuclease A could still be detected in a nonreducing gel (Fig. 1, lane 4). This suggested that oligomerization of ribonuclease A may involve cross-linking *via* isopeptide bonds and that the blocking of free amino groups specifically inhibits isopeptide bond formation without affecting interchain disulfide bond formation. The difference in the mobility of ribonuclease A dimers between lanes 2 and 4 may be due to the difference in disulfide bonds. The presence of inter- and intrachain disulfide bonds in lane 4 may render the peptide

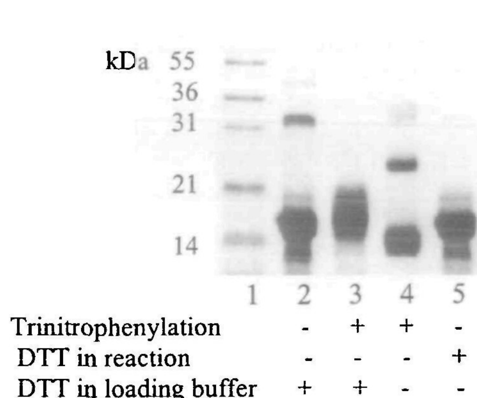

Fig. 1. **Formation of ribonuclease A dimers/oligomers during refolding.** Reduced, denatured ribonuclease A (0.10 mM) was incubated at 37°C for 3 h in 25 mM Tris-HCl buffer (pH 7.6) containing 1.0 mM EDTA, 2.0 mM GSH, and 1.0 mM GSSG (lane 2). The free amino groups of ribonuclease A in lanes 3 and 4 were blocked by chemical modification with trinitrophenyl. The reaction solution in lane 5 contained 10 mM DTT in addition. Before loading into a 12% SDS–polyacrylamide gel, samples in lanes 2 and 3 were treated with reducing loading buffer, while samples in lanes 4 and 5 were treated with nonreducing loading buffer. Lane 1 contains the protein markers.

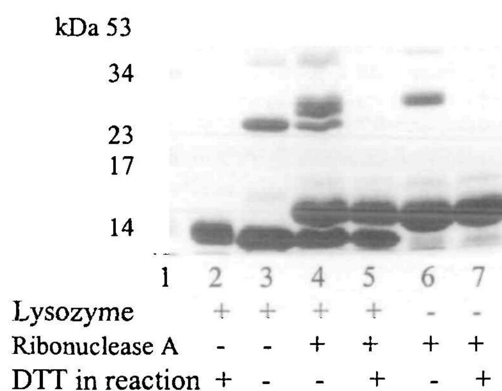

Fig. 2. **Formation of ribonuclease A and lysozyme homo- and heterodimers/oligomers during protein unfolding.** Native ribonuclease A or lysozyme proteins (each 0.1 mM) were incubated for 30 min in 25 mM Tris-HCl (pH 7.6) containing 1.0 mM EDTA, 2.0 mM GSH, and 1.0 mM GSSG. The incubation temperatures were 75°C for lysozyme (lanes 2 and 3), 65°C for ribonuclease A (lanes 6 and 7), and 75°C for the mixture of the two proteins (lanes 4 and 5). The reaction solution in lanes 2, 5, and 7 contained 10 mM DTT in addition. All samples were treated with reducing loading buffer before loading into a 12% SDS–polyacrylamide gel. Lane 1 contains the protein markers.

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chains tighter, thus resulting in their faster mobility in SDS-PAGE. Interestingly, oxidative refolding in the presence of DTT prevented both types of cross-links as revealed by the lack of protein dimers/oligomners in a nonreducing gel (Fig. 1, lane 5). Similar dimer/oligomer formation was observed during oxidative refolding of reduced, denatured lysozyme (data not shown). These observations suggested that these dimers/oligomers contained a covalent cross-link in addition to a disulfide bond. Since blocking of free amino groups by chemical modification prevented such covalent cross-linking during refolding in the absence of oxidative factors, this covalent cross-link may represent an interchain isopeptide bond. Because refolding in the presence of DTT prevented formation of interchain isopeptide bond cross-links, and because blocking of free amino groups failed to inhibit formation of interchain disulfide bonds, we conclude that preformed interchain disulfide bonds are critical in promoting subsequent interchain isopeptide bond cross-links.

Heterogeneous Cross-Linking Following Heteromolecular Interchain Disulfide Bond Formation—During thermal unfolding of native enzymes, the formation of dimers/oligomers was favored at or near the midpoint temperature of the thermal denaturation [*e.g.*, lysozyme at 75°C (Fig. 2, lane 3) and ribonuclease A at 65°C (Fig. 2, lane 6)]. No dimers/oligomers were observed when the unfolding assay was performed in the presence of DTT (Fig. 2, lane 2 *vs.* lane 3 for lysozyme; lane 7 *vs.* lane 6 for ribonuclease A). To further support the contention that preformed interchain disulfide bonds were pivotal for promoting subsequent interchain isopeptide cross-links, we mixed ribonuclease A and lysozyme in a thermal unfolding reaction at lysozyme's midpoint temperature (75°C). Analysis of the reactants by SDS-PAGE revealed that heterodimer/oligomer formation between ribonuclease and lysozyme was as common as homodimer/oligomer formation (Fig. 2, lane 4). As expected, the presence of DTT in the reaction effectively inhibited the formation of not only homodimers/oligomers but also heterodimers/oligomers (Fig. 2, lane 5). These observations further supported the contention that preformed interchain disulfide bonds are crucial for promoting subsequent interchain isopeptide cross-links. Similarly, the presence of heterodimers/oligomers suggested that heterogeneous proteins can also be involved in isopeptide bond cross-links following the formation of heteromolecular interchain disulfide bonds.

Conformational Changes, Including Partial Changes in Protein Secondary Structure, Are Critical for the Cross-Links—Next we studied the effects of unfolding temperature, GSH, GSSG, and pH on protein cross-linking/oligomerization. As suggested by the results shown in Fig. 3, thermal unfolding of ribonuclease A at a temperature lower than the midpoint of its thermal denaturation temperature failed to promote dimer/oligomer formation (Fig. 3, lanes 2, 3, and 4 *vs.* lane 5). It has been reported that changes in the spectra of circular dichroism for ribonuclease A and lysozyme following thermal unfolding are indicative of changes in the fraction of protein secondary structure components (*18–20*). Taken together, these observations suggested that native proteins oligomerize during the unfolding process until their secondary structures have changed to a certain extent. In addition, the lack of GSH and GSSG during the unfolding process attenuated both disulfide bond cross-links (Fig. 3, lane 10) as well as isopeptide bond cross-links (Fig. 3, lane 9), as suggested by a decrease in dimer/oligomer formation. Similarly, the formation of dimers/oligomers as a result of isopeptide bond cross-links was significantly decreased (Fig. 3, lanes 7 *vs.* 5) when

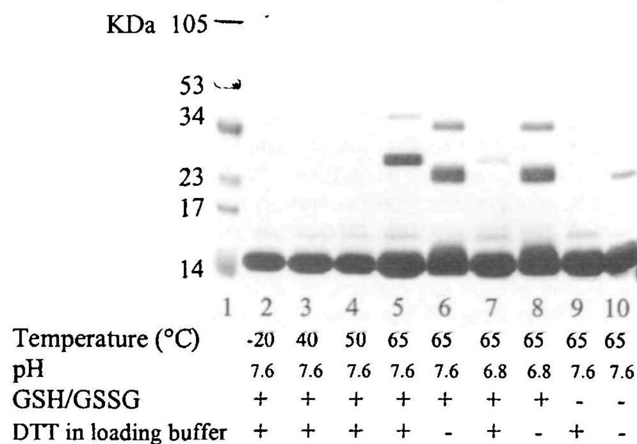

Fig. 3. **Effects of temperature, pH, GSH, and GSSG on formation of dimers/oligomers during the ribonuclease A unfolding process.** Native ribonuclease A protein (0.1 mM) was incubated for 30 min in 25 mM Tris-HCl (pH 7.6) containing 1.0 mM EDTA, 2.0 mM GSH, and 1.0 mM GSSG at 65°C (lanes 5 and 6). Protein was stored at −20°C (lane 2). Protein was unfolded at 40°C (lane3), at 50°C (lane 4), at pH 6.8 (lanes 7 and 8), or without GSH and GSSG (lanes 9 and 10). Before loading into a 12% SDS–polyacrylamide gel, samples in lanes 2, 3, 4, 5, 7, and 9 were treated with reducing loading buffer, while samples in lane 6, 8, and 10 were treated with non-reducing loading buffer. Lane 1 contains the protein markers.

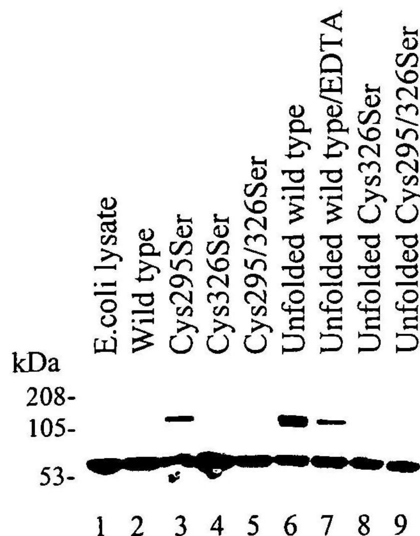

Fig. 4. **Western blotting of purified and thermal unfolded PDIs.** All samples were reduced and loaded into a 10% SDS–polyacrylamide gel. PDI polyclonal antibody was used to probe the blot. 1, lysate of *Escherichia coli* cells containing wild-type PDI; 2, purified wild type; 3, purified Cys295Ser mutant; 4, purified Cys326Ser mutant; 5, purified Cys295/326Ser mutant; 6, thermal unfolded wild type; 7, thermal unfolded wild type containing 10 mM EDTA; 8, thermal unfolded Cys326Ser mutant; 9, thermal unfolded Cys295/326Ser mutant.

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unfolding of ribonuclease A was carried out at pH < 7.0. In contrast, disulfide bond cross-linking was not affected by pH (Fig. 3, lanes 8 *vs.* 6). Similarly, thermal unfolding of bovine PDI at pH 8.5 completely polymerized the protein, as revealed by the complete absence of a monomeric band (data not shown). Because the nucleophilic charge of the amino groups in a protein weakens at an acidic pH, it is likely that isopeptide bond formation fails at pH < 7.0. It is apparent from the results presented here that cross-linking/oligomerization of proteins is mediated in three steps: (i) protein conformational changes, including partial changes in secondary structure, (ii) formation of interchain disulfide bonds, and (iii) interchain isopeptide bond cross-linking.

Point Mutation (Cys326Ser) Abrogates PDI's Ability to Cross-Link into Homodimers—To further substantiate the three-step process for dimer/oligomer formation, we extended our observations with PDI. In purified preparations of PDI protein, oligomeric bands in addition to the major 55-kDa band have been consistently observed by several investigators (*21–23*). The molecular mechanism underlying PDI's oligomerization is not well understood. Recently, we discovered that bovine PDI has calcium-dependent transglutaminase activity which catalyzes the formation of isopeptide bonds (*24*). In addition to four cysteine residues in PDI's two thioredoxin-like regions, two additional cysteines (*25*) (Cys295 and Cys326) are highly conserved among various members of the mammalian PDI family. We therefore postulated that Cys295 and Cys326 may form a disulfide bond in native PDI molecules, and that breakage of this bond might enable PDI to form interchain disulfide bonds that in turn may lead to formation of interchain isopeptide bonds. To test this hypothesis, we generated three PDI point mutants containing a serine residue in place of either or both of the cysteines (Cys295Ser, Cys326Ser, and Cys-295/326Ser). Western blotting of purified recombinant proteins revealed that PDI completely lost its ability to form dimers when the Cys326 residue was replaced with serine (Fig. 4, lanes 4 and 5). Conversely, the Cys295Ser mutant showed a much stronger ability to form cross-linked dimers under similar conditions (Fig. 4, lane 3). The thermal unfolding of wild-type PDI protein showed a high propensity toward dimer formation (Fig. 4, lane 6), whereas the Cys326Ser and Cys295/326Ser mutants completely failed to form dimers under these conditions (Fig. 4, lanes 8 and 9). It has been previously reported that the 21-kDa C-terminal fragment of human PDI (containing Cys326 but not Cys295) forms a dimer by an interchain disulfide bond through Cys326 (*26*). Unlike ribonuclease A and lysozyme, PDI has calcium-dependent transglutaminase activity, which catalyzes the formation of isopeptide bonds (*24*). So PDI's ability to form dimers/oligomers can be influenced by the presence of calcium ions. EDTA could block the catalytic reaction but not the noncatalytic reaction like that of ribonuclease A or lysozyme. Thus, the cross-linking of PDI was slightly inhibited in the presence of EDTA, as revealed by attenuation in the dimer band (Fig. 4, lane 7 *vs.* lane 6). However, as was true with ribonuclease A, blocking PDI's free amino groups with trinitrobenzenesulfonin almost completely inhibited dimer formation (data not shown). These observations suggested that Cys295 and Cys326 residues formed a disulfide bond in native PDI molecules. Conformational changes induced by heating or mutation of Cys295 residue may have freed Cys326, enabling it to form an interchain disulfide bond that is critical in promoting subsequent isopeptide bond cross-links.

Our *in vitro* experiment thermodynamically suggests that protein cross-linking/oligomerization can happen *in vivo* and that it may be kinetically catalyzed in one, two, or three steps by a specific enzyme such as transglutaminase. One way enzymes efficiently catalyze conversion of substrate molecules into products during vital reactions is by selectively lowering the energies of activation. Kinetically, the preformed disulfide bonds can also reduce activation energies to favor formation of isopeptide bonds and other interactions, just like an enzyme does. Thermodynamically, these processes are driven by a change in free energy (ΔG). Although ΔG is positive during structural change, later interactions may render it negative, and net ΔG is negative for protein cross-linking/oligomerization. From a thermodynamic point of view, the ΔG for a reaction resulting in a covalent bond formation would be more favorable if the second product formed is NH_3 or H_2O. Accordingly, the involvement of glutamic acid or glutamine is likely to be favored in covalent bond formation following disulfide bond formation. Studies to delineate the precise nature of amino acids involved in cross-links during refolding/unfolding of PDI protein are currently in progress in our laboratory.

Protein folding has been studied for more than half a century, and disulfide bonds are believed to be the only common type of covalent bonds formed during protein folding. The results presented here reveal an interesting aspect of the protein folding process. Thus, the interchain disulfide bonds can promote the formation of interchain isopeptide bonds during the protein refolding and unfolding process. Theoretically, this phenomenon could also occur within the same polypeptide chain. It is likely that the formation of intrachain isopeptide bonds follows the formation of the intrachain disulfide bonds during protein folding. However, due to the lack of techniques for detecting such intrachain bonds, their existence has not been formally proven. There is no evidence that intrachain isopeptide bonds exist in protein crystal structure. In the ubiquitin-proteasome proteolysis pathway, ubiquitin molecules are linked via an interchain isopeptide bond between peptide chains. This fact was discovered by biochemical methods (*27*) and confirmed by analysis of crystal structure (*28*). For this reason, it might be worthwhile to reexamine the crystal structures of proteins that may contain intrachain isopeptide bonds.

Similarly, the inclusion bodies formed as a result of eukaryotic cDNA expression in *Escherichia coli* exist in reduced form (*29*). Unlike those formed in the course of protein deposit diseases, the inclusion bodies do not contain cross-linked aggregates of the expressed protein. It is possible that reducing conditions in the cytoplasm of *E. coli* cells may decrease the formation of interchain disulfide bonds that are pivotal for subsequent formation of isopeptide bonds, as revealed by the present results.

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