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Concurrent Translocation of Multiple Polypeptide Chains through the Proteasomal Degradation Channel*

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The proteasome can actively unfold proteins by sequentially unraveling their substrates from the attachment point of the degradation signal. To investigate the steric constraints imposed on substrate proteins during their degradation by the proteasome, we constructed a model protein in which specific parts of the polypeptide chain were covalently connected through disulfide bridges. The cross-linked model proteins were fully degraded by the proteasome, but two or more cross-links retarded the degradation slightly. These results suggest that the pore of the proteasome allows the concurrent passage of at least three stretches of a polypeptide chain. A degradation channel that can tolerate some steric bulk may reconcile the two opposing needs for degradation that is compartmentalized to avoid aberrant proteolysis yet able to handle a range of substrates of various sizes.

Protein degradation is a critical part of cellular regulation (1). In eukaryotic cells, a multicomponent protease called the proteasome is responsible for the turnover of short-lived regulatory proteins, the removal of abnormal polypeptides, and the production of peptides for antigen presentation (2). Degradation of short-lived regulatory proteins is essential for a wide range of cellular functions including cell cycle control and signal transduction (3). Failure to degrade aggregates of misfolded proteins can lead to disease such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (4). Degradation by the proteasome usually involves two consecutive steps: targeting of the substrate for degradation by the attachment of polyubiquitin chains and degradation of the tagged protein by the proteasome with concomitant release of ubiquitin (5).

The active sites of proteolysis of the proteasome by themselves show little substrate specificity. Specificity of degradation is achieved by sequestering the proteolytic sites within the structure and tightly controlling access. The three-dimensional structure of the proteasome has been determined by x-ray crystallography and electron microscopy (6–11). The proteasome consists of a central proteolytic core particle with regulatory caps at either end of it. The core particle is made of two copies of seven different α-subunits and seven different β-subunits. The subunits are arranged in four heptameric rings, which are stacked on top of each other to form a cylindrical particle. The rings form a central β-chamber and two α-chambers, one at each end of the particle (2). The proteolytic sites are located in the β-chamber and are accessible only through a central channel that runs along the long axis of the particle. The channel has narrow constrictions at the entrance and exit of the α-chamber (2). In the isolated yeast core particle, the entrance to the degradation channel is blocked by the N termini of the α-subunits (7). The channel opens when the regulatory caps bind to the core (12). The caps consist of 17 subunits, six of which have ATPase activity, and contact the core particle through the ATPase subunits (13, 14).

At its narrowest point, the opened degradation channel is ~13 Å wide (11, 12). This constriction is too small for folded proteins to fit through it, and substrate proteins must unfold to gain access to the proteolytic sites. Preventing unfolding of a substrate protein protects it from degradation (15). It is now thought that the ATPase subunits in the cap can actively unfold protein substrates (16). Studies with model proteins with N-terminal ubiquitination sites suggested that the unfolding is induced by the unraveling of the substrate from its N terminus by the proteasome (17). In model proteins with more than one folded domain, the proteasome first unfolded and degraded the domain at the N terminus adjacent to the ubiquitination site and then the next domain in the protein (17). Together, these findings conjure an image of the proteasome threading a single polypeptide chain through the degradation channel as it degrades its substrate sequentially. This image is consistent with the small size of the entrance to the degradation channel.

The steric constraints of the proteasome degradation channel raise some questions. For example, how are proteins degraded that contain large modification such as *O*-linked carbohydrates? Can a multidomain protein be degraded from its center so that multiple polypeptide chains are threaded through the degradation channel simultaneously? The ubiquitination sites for a small number of substrates have been defined. For some of them, ubiquitination occurs near the N terminus, as is the case for proteins targeted for degradation by the N-end rule (18), for IκBα (19–21) and for cyclins (22–24). Analogous to the results from model protein studies (17), it seems likely that these proteins will be sequentially degraded from their N termini. However, some proteasome substrates are ubiquitinated on internal sites. Examples are NFκB (25–27), SnoN (28), $p27^{Kip1}$ (29), and Spt23p and Mga2p (30, 31). Are these proteins also degraded from their ends, or can degradation begin from the internal ubiquitination site with the formation of a loop that is fed into the proteasomal degradation channel? This question is particularly pertinent for the activation of the yeast transcription factors Spt23p and Mga2p by the proteasome (30, 31). These transcription factors are anchored to membranes through their C termini. During activation, the

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proteasome degrades the C-terminal part of the transcription factors to release an N-terminal fragment (30, 31). Because the C termini of the transcription factors are blocked by the membrane and the N termini are released intact, degradation must begin from the middle of the precursor proteins.

To investigate the steric constraints imposed on substrate proteins by the proteasome structure, we determined whether the degradation channel is flexible enough to allow more than one polypeptide to pass through it at the same time. We constructed a model protein in which specific parts of the polypeptide chain were covalently connected through disulfide bridges. We found that cross-linked model proteins were fully degraded by the proteasome although the presence of two or more disulfide bridges retarded degradation. Therefore, we suggest that the pore of the proteasome permits the concurrent passage of at least three polypeptide chains.

MATERIALS AND METHODS

Substrate Proteins—Protease substrates were derived from barnase, a ribonuclease from *Bacillus amyloliquefaciens* (32), and dihydrofolate reductase (DHFR)[1] from *Escherichia coli* (33). The two proteins were linked in-frame, with barnase at the N terminus followed by DHFR. Ubiquitin and a 40-amino acid linker derived from the *E. coli* lac repressor were attached to the N terminus of barnase to target the substrate protein to the proteasome by the N-end rule pathway (Fig. 1) (15, 34). In reticulocyte lysate, the N-terminal ubiquitin is rapidly cleaved, and the remaining protein is ubiquitinated on two lysine residues in the 40-amino acid linker at the N termini of the fusion proteins (35).

Wild-type barnase, the ubiquitin domain, and the targeting linker do not contain cysteine residues. Two cysteine residues in DHFR were mutated to alanine. The changes did not affect the affinity of DHFR toward methotrexate significantly (the dissociation constant is ~20 nM in the buffer employed for the degradation assay). We introduced three different disulfide bonds into barnase to covalently link neighboring β-strands within the five-stranded antiparallel β-sheet of barnase (36) (Fig. 1*B*). The structures of barnase proteins containing these cross-links were determined by x-ray crystallography and found to be almost identical to that of wild-type barnase (37). We also constructed substrate proteins containing all combinations of two and three disulfide bridges.

The genes for the various proteasome substrates were assembled using standard molecular biology techniques in pGEM-3Zf(+) vectors (Promega Corp.), and the constructs were verified by DNA sequencing. Radioactive proteins were expressed from a T7 promoter by *in vitro* transcription and translation in *E. coli* S30 extract supplemented with [^{35}S]methionine. Neighboring cysteine residues were induced to form disulfide bridges by oxidation with 10 mM ferricyanide ($K_3Fe(CN)_6$) for 10 min at room temperature. Under these conditions disulfide bridge formation is complete, and no unreacted cysteine residues could be detected by modification with the sulfhydryl-reactive reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (SDSM) followed by SDS-PAGE (36) (data not shown). The proteins were partially purified by high speed centrifugation before cross-linking and by ammonium sulfate precipitation after cross-linking as described previously (36).

Proteasome Degradation Assay—Degradation by the proteasome was assayed in rabbit reticulocyte lysate essentially as described (17, 38) except that the lysate was dialyzed against 10 mM Tris-Cl, pH 8.0, prior to the degradation assay. Substrate proteins produced by *in vitro* translation were resuspended in 5 μl of buffer (25% (v/v) glycerol, 25 mM $MgCl_2$, 0.25 M Tris-Cl, pH 7.4), added to 20 μl of ATP-depleted reticulocyte, and incubated for 20 min at 25 °C to allow removal of the N-terminal ubiquitin by deubiquitinating enzymes (38). Ubiquitination and degradation were initiated by addition of ATP and an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase; final concentrations), and incubation continued at 25 °C. At designated time points, 2.4-μl aliquots were transferred to 20 μl of ice-cold 5% trichloroacetic acid, and the trichloroacetic acid-insoluble fractions were analyzed by SDS-PAGE and electronic autoradiography (17). For the degradation of reduced substrate, protein substrate was preincubated with 10 mM DTT for 2 min. Degradation was strictly dependent on a destabilizing N-terminal amino acid (arginine *versus* methionine) and ATP and was inhibited between 2- and 5-fold by the dipeptide Arg-Ala (10 mM, data not shown), which suppresses ubiquitination (39).

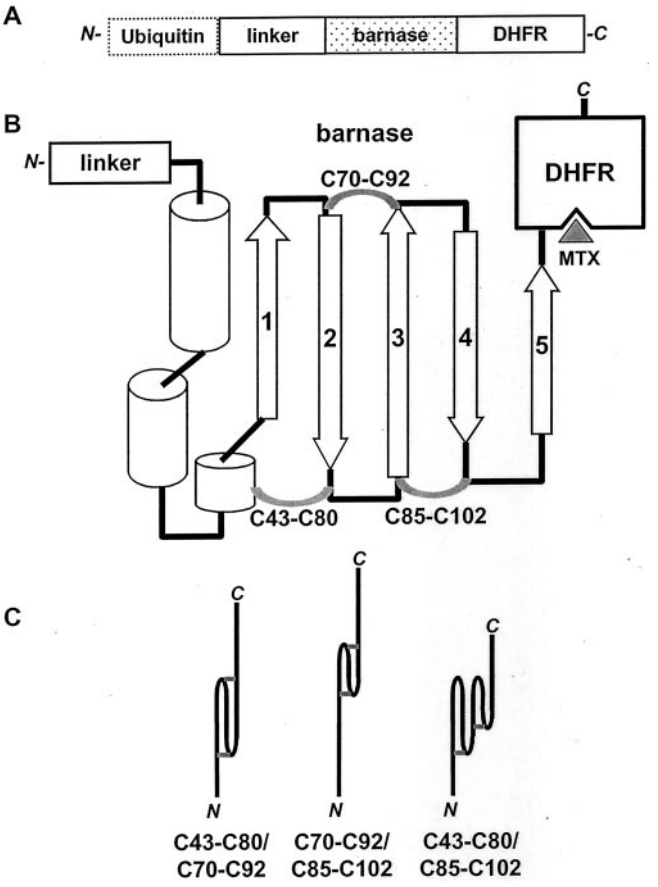

FIG. 1. **Schematic representations of the substrate protein.** *A*, linear representation of substrate construct. The N-terminal ubiquitin domain is cleaved immediately in reticulocyte lysate. *B*, barnase domain depicted in a wire diagram. α-Helices are shown as *cylinders*, β-strands as *arrows*. Disulfide bridges are drawn in *gray* and labeled with the positions of the participating cysteine residues. *MTX*, methotrexate. *C*, the residual structure introduced into barnase by two disulfide bridges at different positions when the protein is fully extended from its termini.

RESULTS

Substrate Proteins—We investigated the degradation of proteasome substrates in which loops were introduced into the polypeptide chain by disulfide bridges. The substrate proteins consisted of three parts: an N-terminal targeting region, a barnase domain, and finally a DHFR domain (Fig. 1). To prevent specific parts of the substrate protein from unfolding, we introduced covalent cross-links into the barnase domain by mutating pairs of residues to cysteine at positions that allow disulfide bridge formation upon oxidation (36, 37). The disulfide bridges were introduced at three different positions, Cys^{43}–Cys^{80}, Cys^{70}–Cys^{92}, and Cys^{85}–Cys^{102} (the numbering refers to the residue position within the barnase domain). In addition to fusion proteins with these three single disulfide bridges, we constructed all combinations of double disulfide bridge mutants and a fusion protein with all three disulfide bridges present simultaneously. After synthesis of radioactively labeled fusion proteins by *in vitro* transcription and translation, disulfide bridge formation was induced by oxidation with ferricyanide, as described previously (36). Disulfide bridge formation was complete, and no unreacted cysteine residues could be detected with the sulfhydryl-reactive

[1] The abbreviations used are: DHFR, dihydrofolate reductase; SDSM, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; DTT, dithiothreitol.

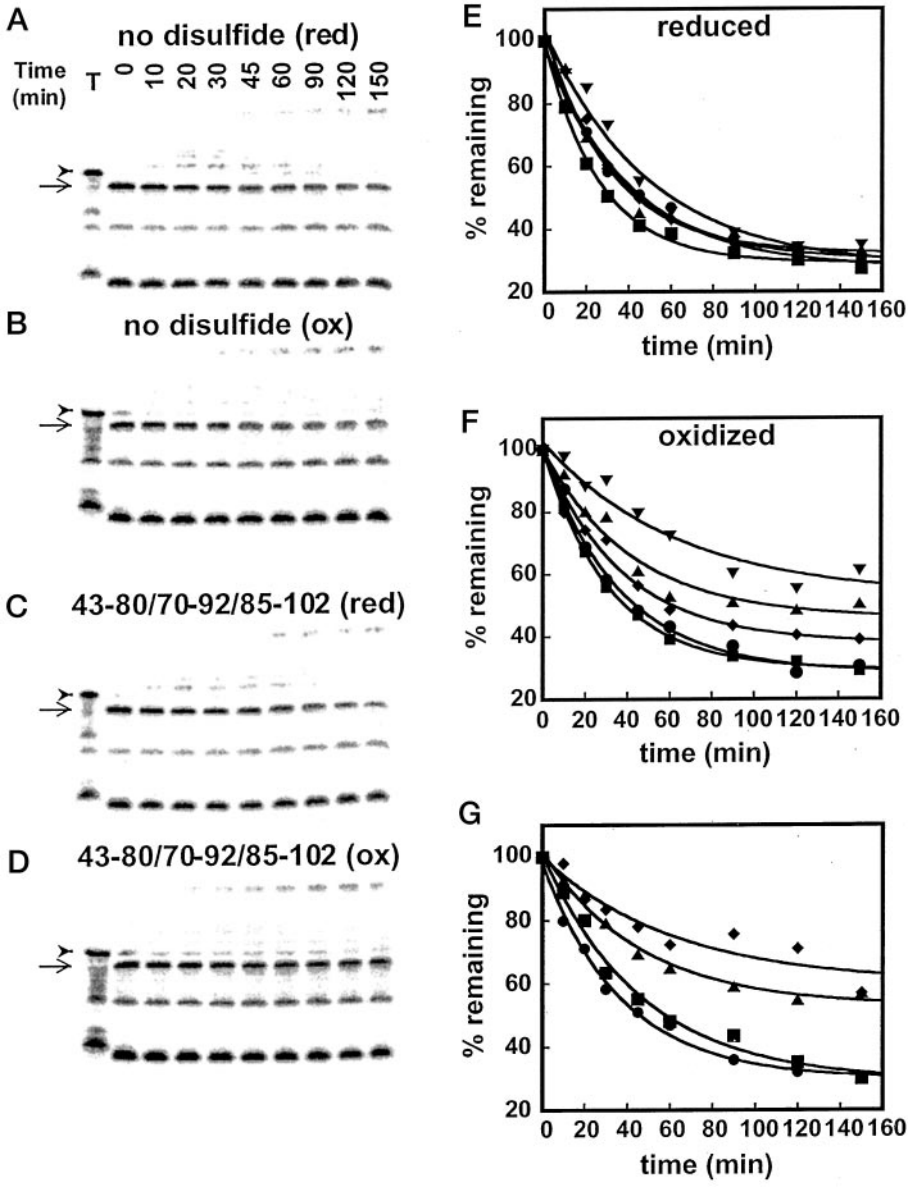

FIG. 2. **Only extensive cross-linking of substrate proteins inhibits their degradation by the proteasome.** *A–D*, autoradiograms of SDS-PAGE gels with degradation assays of a substrate protein without disulfide bridges and a substrate protein with three disulfide bridges. *A* and *B,* substrate without disulfide bridges. *C* and *D*, substrate containing three pairs of Cys residues able to form disulfide bridges upon oxidation (Cys^{43}–Cys^{80}/Cys^{70}–Cys^{92}/Cys^{85}–Cys^{102}). The substrate proteins were either reduced with DTT (*A, C*) or oxidized with ferricyanide (*B, D*) before the beginning of the assay. The total lane (*T*) contains the untreated ubiquitin-fusion protein (*marked by arrowhead*) produced in a cell-free *E. coli* translation system. In the zero time lane the N-terminal ubiquitin tag has been completely removed (*marked by arrow*) during the preincubation in ATP-depleted reticulocyte lysate. *E,* quantification of degradation assays of substrate proteins with no (●, no disulfide), one (■, Cys^{43}–Cys^{80}), two (♦, Cys^{43}–Cys^{80}/Cys^{70}–Cys^{92}; ▲, Cys^{43}–Cys^{80}/Cys^{85}–Cys^{102}), and three (▼, Cys^{43}–Cys^{80}/Cys^{70}–Cys^{92}/Cys^{85}–Cys^{102}) disulfide bridges after reduction with DTT. *F,* degradation of the same substrate proteins as in *E* after oxidation with ferricyanide. *G*, substrate unfolding is not rate-determining for degradation. Destabilizing the barnase domain in a substrate protein containing three disulfide bridges by the mutation $Ile^{25} \rightarrow Ala$ does not affect degradation rates. ●, substrate lacking disulfide bridges, reduced; ▲, substrate with three disulfide bridges, oxidized; ■, substrate with three disulfide bridges and the destabilizing mutation $Ile^{25} \rightarrow Ala$, reduced; ♦, substrate with three disulfide bridges and the destabilizing mutation $Ile^{25} \rightarrow Ala$, oxidized.

reagent SDSM (data not shown). The constructs were targeted to the proteasome in reticulocyte lysate by the N-end rule pathway (34), and ubiquitination occurred on two lysine residues in a 40-amino acid linker at the N termini of the fusion proteins (35).

A Polypeptide Loop Can Be Translocated through the Proteasome Channel—We first sought to determine whether a polypeptide loop could be translocated through the degradation channel. To address this question, we measured the effect of intramolecular disulfide bridges within the barnase domain on degradation of the fusion protein by the proteasome. Because disulfide bonds are covalent cross-links, they persist after a protein is unfolded and introduce a permanent polypeptide loop into the primary structure (see below). Radiolabeled fusion proteins were synthesized by coupled *in vitro* transcription and translation in *E. coli* S30 extract, and disulfide bridge formation was induced by oxidation (36). After partial purification, the proteins were incubated in reticulocyte lysate, and their degradation was monitored by SDS-PAGE and electronic autoradiography (Fig. 2). Degradation occurred with the same half-time of 25–30 min for fusion proteins without cross-links and those containing a single disulfide bridge (Fig. 2*F*). The same degradation kinetics were also obtained when disulfide bridges were reduced by DTT before assaying for degradation. The proteasome degrades proteins consisting of multiple domains sequentially beginning with the degradation signal (17). Therefore, if the cross-links blocked complete degradation, we would expect proteolysis of the substrates up to the first disulfide bridge and the accumulation of the remainder of the polypeptide chain. However, no partially degraded substrate proteins could be detected (Fig. 2, *B* and *D*). Together, these results suggest that single cross-links within a substrate protein do not affect degradation by the proteasome and that the degradation channel through the proteasome can tolerate at least one loop in a polypeptide chain.

Residual Structure Retards Translocation of Unfolded Polypeptide—Next, we tested how much steric bulk in the substrate protein can be tolerated by the proteasomal degradation channel. For this purpose, we increased the number of disulfide bonds in barnase. As substrates were forced to retain more residual structure during degradation, both the rates and extent of degradation decreased.

In a sequentially degraded substrate, the amount of steric bulk introduced by disulfide bridges depends on the location of the cross-links within the polypeptide chain. We analyzed three

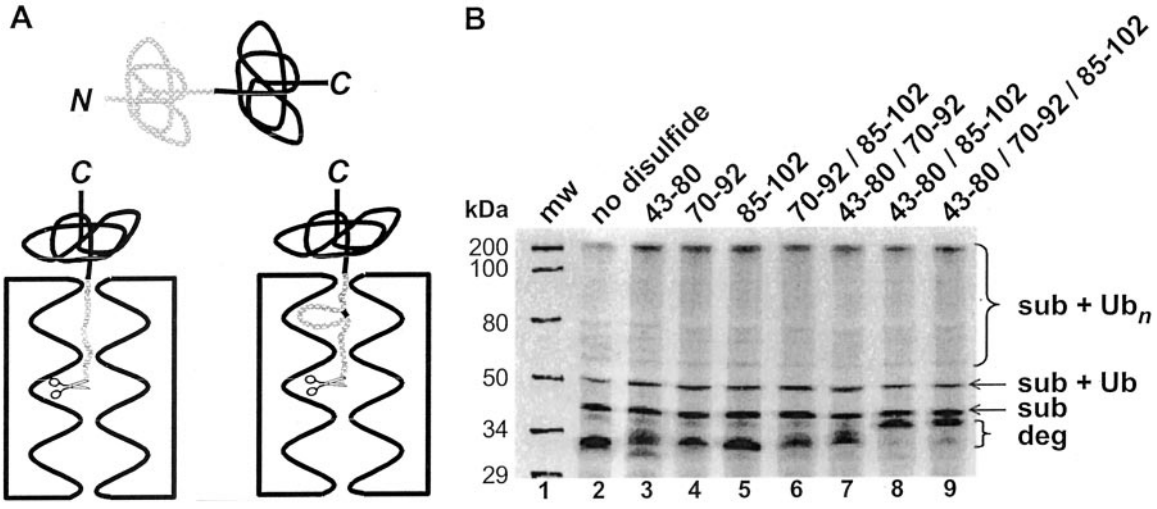

FIG. 3. **Effect of disulfide bridge cross-links on the size of partial degradation products.** *A*, proposed conformation of partially degraded substrate proteins in the absence and presence of cross-links. Partial degradation occurs when the C-terminal DHFR domain of the substrate is stabilized against unfolding with methotrexate. Methotrexate-stabilized DHFR is shown in *black*, and barnase is shown in *gray*. *B*, autoradiogram of an SDS-PAGE gel with degradation reactions of substrate proteins whose DHFR domain is stabilized against unfolding and degradation by methotrexate binding. The substrate proteins differ in the number and position of disulfide bridges. The locations of the cross-links are indicated at the *top* of each lane. Oxidized proteins were degraded in reticulocyte lysate at 25 °C for 2~3 h. *sub* indicates the actual deubiquitinated substrate; *sub + Ub*, uncleaved ubiquitin fusion protein and monoubiquitinated species; *sub + Ub$_n$*, polyubiquitinated protein; *deg*, degradation end product; *mw*, molecular weight markers.

different mutants containing two disulfide bridges. In two mutants, Cys^{43}–Cys^{80}/Cys^{70}–Cys^{92} and Cys^{70}–Cys^{92}/Cys^{85}–Cys^{102}, the first cysteine in the amino acid sequence forms a cross-link with the third cysteine, and the second cysteine forms a cross-link with the fourth cysteine. In both of these mutants, the cysteine residues are spaced so that the second cross-link does not introduce a new loop into the polypeptide chain during sequential degradation from the N terminus (Fig. 1*C*). When these proteins are degraded from their N termini, three strands of the polypeptide chain have to pass through the degradation channel simultaneously. Degradation rates of Cys^{43}–Cys^{80}/Cys^{70}–Cys^{92} and Cys^{70}–Cys^{92}/Cys^{85}–Cys^{102} were similar to those of substrates lacking disulfide bridges (Fig. 2 and data not shown).

In a third mutant, Cys^{43}–Cys^{80}/Cys^{85}–Cys^{102}, the first two cysteine residues in the polypeptide chain cross-link with each other, and the third and fourth cysteine cross-link with each other. In this mutant, the two disulfide bridges introduce separate loops into the polypeptide. Both loops have to pass through the degradation channel simultaneously during sequential degradation from the N terminus (Fig. 1*C*). The cross-links introduced in this mutant reduced the degradation rate to a small but reproducible extent (Fig. 2*F*). Introduction of a third disulfide bridge to create the mutant Cys^{43}–Cys^{80}/Cys^{70}–Cys^{92}/Cys^{85}–Cys^{102} reduced degradation rates further (Fig. 2*F*). In addition, the extent of degradation was reduced to ~60% of that of a substrate lacking cross-links. For all proteins, reducing the disulfide bridges with DTT restored the rates and extent of degradation to that of substrates lacking cysteine residues (Fig. 2*E*). It is unlikely that the disulfide bridges affected the ubiquitination step because the ubiquitination sites are far away from the folded barnase domain and none of the single disulfide bridges affected degradation (see also below).

Substrate Unfolding Is Not Rate-determining for Degradation—Disulfide bridges can affect the stability of the folded substrate protein against global unfolding (40). Thus, the inhibition of degradation induced by the cross-links could be caused by the stabilization of the native protein against unfolding rather than by the residual structure in denatured barnase. We could differentiate between these two cases by introducing an additional destabilizing mutation in the protein containing three disulfide bridges. The $Ile^{25} \rightarrow Ala$ mutation destabilizes barnase by 3.5 kcal/mol and accelerates unfolding rates to a similar extent (Ref. 41 and data not shown). The mutation did not accelerate degradation of the substrate containing three disulfide bridges, independently of whether these disulfide bridges were formed or not (Fig. 2*G*). This result suggests that the decreased degradation of the triple disulfide mutant is not due to any stabilization of the native state. Instead, the degradation is presumably inhibited by the residual structure in the unfolded substrate exerting steric hindrance on the translocation of the polypeptide.

Disulfide Bonds Remain Intact during Degradation—The degradation of disulfide cross-linked substrates could also occur if the proteasome were able to break disulfide bridges during degradation. Degradation of substrates with multiple disulfide bridges would then be delayed because of the time it takes to break multiple cross-links. To test for this possibility, we compared the sizes of end products produced when the C-terminal DHFR domain of the substrate proteins was stabilized by ligand binding. Methotrexate binding stabilizes DHFR against unfolding and protects it from proteolysis by the proteasome (15). Stabilization of the DHFR domain allows degradation of the N-terminal portion of the substrate including part of the barnase domain. Degradation stops at a point in the polypeptide chain that is ~90 amino acids upstream of the DHFR domain (17) (Fig. 3*B*, *lane 2*). The undegraded 90-amino acid tail must stretch from the proteolytic sites to the entrance to the degradation channel where the folded DHFR domain becomes stuck. All the residues involved in cross-links are contained within this tail. When the structure of the undegraded tail becomes restricted by cross-links, more than 90 amino acids may be required to bridge the distance between proteolytic sites and the entrance to the degradation channel (Fig. 3*A*). Thus, intact disulfide bridges in the barnase domain could cause the accumulation of degradation products with longer undegraded tails when DHFR is stabilized. This is indeed what we observed. As increasing amounts of disulfide bridges are introduced into the substrate protein, the mobility of the degradation end product in SDS-PAGE decreases (Fig. 3*B*). For the constructs containing two or three disulfide

bridges, no or only very little degradation product of the size found for precursors lacking disulfide bridges can be detected (Fig. 3*B*, compare *lanes 8* and *9* with *lane 2*). This result indicates that disulfide formation was complete and that the cross-links are maintained throughout the degradation reaction. When disulfide bonds were reduced before degradation, the end products of degradation all showed the same mobility on SDS-PAGE as the substrate lacking cysteine residues (data not shown). Together these results show that the proteasome does not reduce the disulfide bonds during degradation. In addition, the findings demonstrate that disulfide bond formation did not interfere with proteasome targeting because all substrate proteins were processed irrespective of the number of disulfide bridges.

DISCUSSION

Can multiple strands of a polypeptide chain pass through the degradation channel in the proteasome simultaneously? Crystal structures of the yeast proteasome core particle in complex with the caps show that the axial pore has a diameter of ~13 Å (11, 12, 14). The degradation channel in the archebacterial proteasome has restrictions that are smaller than 20 Å as judged by the crystal structure (6) and the observation that a gold particle with a diameter of ~ 20Å attached to a substrate protein could not enter the archebacterial proteasome (42). These size restrictions are presumably the reason that even small folded proteins such as DHFR cannot be degraded without prior unfolding (15, 17). Three extended polypeptide chains packed against each other are expected to have diameters in the range of 13–20 Å. We found that forcing three polypeptide chains to pass through the degradation channel by introducing one or two disulfide bridges into a substrate protein does not affect degradation rates (Fig. 2*F*). In these experiments, the degradation rate was not limited by the unfolding of the substrate protein (Fig. 2*G*) but presumably by substrate enzyme encounter because of the small concentrations of both substrate and protease. Therefore, we cannot rule out that the steric bulk introduced into the substrate would have a small effect if translocation were rate-determining for degradation. Increasing the amount of residual structure in the translocating substrate protein further by positioning two disulfide bridges appropriately or by introducing a third disulfide bridge leads to a small but reproducible decrease in degradation rates (Fig. 2*F*). This result suggests that the proteasome channel is sufficiently wide or flexible for the concurrent passage of five polypeptide chains. A similar result was obtained for the bacterial ATP-dependent protease ClpXP. ClpXP degrades dimeric P22 Arc repressor bearing a C-terminal SsrA tag with similar efficiencies whether or not the two subunits are cross-linked with a disulfide bridge (43). An example of an unrelated protein translocation pore that allows concomitant passage of five polypeptide strands is the mitochondrial protein import channel (36, 44).

There are several situations in which more than one polypeptide chain may pass simultaneously through the proteasome degradation channel in the cell. Degradation from an internal site may be responsible for the activation of the yeast transcription factors Spt23p and Mga2p (30). Mga2p and Spt23p are activated when degradation of their C-terminal portions by the proteasome releases their N-terminal DNA binding and activation domains (30). The C termini of Spt23p and Mga2p are anchored in the endoplasmic reticulum. Therefore, processing of these transcription factors has to begin either with an endoproteolytic cleavage by another protease or by the insertion of a loop of the polypeptide chain through the proteasomal degradation channel. Our results suggest that there are no steric constraints to the insertion of two polypeptide chains into the proteasome and therefore no structural requirement of an endoproteolytic cut. Sequential degradation of a protein from an internal ubiquitination site appears possible.

The ability of the proteasome to degrade a protein from an internal site may also be important for the removal of misfolded proteins. Furthermore, a flexible degradation channel will allow the proteolysis of proteins carrying larger modifications, such as *O*-linked carbohydrates. Thus, a degradation channel that can tolerate some steric bulk may reconcile the two opposing needs for a cellular degradation machine that is compartmentalized to avoid aberrant degradation yet able to handle a range of substrates of various sizes.

Another situation in which more than one polypeptide chain may pass through the degradation channel simultaneously occurs when ubiquitin modifications are degraded together with the protease substrate. In yeast, the deubiquitinating enzyme Doa4 is associated with the 26 S proteasome (45, 46). Yeast cells lacking Doa4 are significantly depleted of ubiquitin, and genetic evidence shows that the proteasome is at least partially responsible for the degradation of ubiquitin (45, 46). A similar situation occurs when the deubiquitinating activity is inhibited by ubiquitin-aldehyde (47). Polyubiquitin chains are assembled through isopeptide bonds between a lysine side chain in the substrate or a ubiquitin moiety already attached to the substrate and the C-terminal carboxyl group of the next ubiquitin. Therefore, sequential degradation of a substrate protein without prior release of the ubiquitin moieties would require several polypeptide chains to pass through the degradation channel simultaneously.

Finally, our results may also have implications on the conformation of the polypeptide chain during translocation. When a protease-resistant domain stops the sequential degradation of a multidomain protein, the last proteolytic cleavage occurs some 90 amino acids for the beginning of the resistant domain (17). In a fully extended conformation, a 90-amino acid polypeptide is longer than would be required to bridge the distance from the entrance of the degradation channel to the proteolytic sites. One possible explanation for such a long undegraded chain is that the unfolded substrate protein is pushed through the proteasome channel from the entrance. The protein then fills up the degradation channel until the front end is pushed into catalytic chamber. An alternative explanation would be that polypeptide chain follows a defined, if complex, path from the regulatory caps to the catalytic sites of the β-rings. Our results appear to rule out the first scenario because that scenario predicts that cross-links in the polypeptide chain do not affect the length of the undegraded tail.

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