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Loops in the Central Channel of ClpA Chaperone Mediate Protein Binding, Unfolding, and Translocation

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Summary

The cylindrical Hsp100 chaperone ClpA mediates ATP-dependent unfolding of substrate proteins bearing "tag" sequences, such as the 11-residue ssrA sequence appended to proteins translationally stalled at ribosomes. Unfolding is coupled to translocation through a central channel into the associating protease, ClpP. To explore the topology and mechanism of ClpA action, we carried out chemical crosslinking and functional studies. Whereas a tag from RepA protein crosslinked proximally to the flexible N domains, the ssrA sequence in GFP-ssrA crosslinked distally in the channel to a segment of the distal ATPase domain (D2). Single substitutions placed in this D2 loop, and also in two apparently cooperating proximal (D1) loops, abolished binding of ssrA substrates and unfolded proteins lacking tags and blocked unfolding of GFP-RepA. Additionally, a substitution adjoining the D2 loop allowed binding of ssrA proteins but impaired their translocation. This loop, as in homologous nucleic-acid translocases, may bind substrates proximally and, coupled with ATP hydrolysis, translocate them distally, exerting mechanical force that mediates unfolding.

Introduction

Hexameric ring assemblies containing one or two AAA+ ATPase domains per subunit have been recognized to mediate ATP-dependent protein disassembly and unfolding in a variety of contexts in the cell (Sauer et al., 2004). For example, the NEM-sensitive factor, NSF, mediates dissociation of coiled-coil helices forming SNARE complexes during vesicle fusion (Söllner et al., 1993), while the p97 component plays a key role in the dislocation of proteins from membranes, e.g., during retrotranslocation of misfolded proteins from the ER (Ye et al., 2001). Moreover, a general role has been identified for the hetero-oligomeric ring of AAA+ ATPases lying at the base of the 19S proteasome cap structure in unfolding and translocating proteins into the 20S proteasome core particle (Glickman et al., 1998). In prokaryotic cells, analogous unfolding actions are exerted by such Hsp100 chaperone components as ClpX and ClpA, which associate with the proteolytic ring assembly ClpP (Hoskins et al., 2001). The nature of substrate recognition by these chaperones and how subsequent ATP-driven unfolding and translocation are mediated by them remain unclear.

Both ClpX and ClpA typically recognize terminal sequences, or "tags," in substrate proteins (Keiler et al., 1996; Hoskins et al., 2000a; Flynn et al., 2003). For example, both recognize the C-terminal ssrA undecapeptide added at the ribosome to translationally stalled nascent chains (Keiler et al., 1996; Flynn et al., 2001). Analogously, both chaperones are able to bind the fusion protein GFP-ssrA, unfold it, and translocate it into ClpP, where it is degraded (Weber-Ban et al., 1999; Kim et al., 2000). In the case of ClpX, an adaptor protein, the SspB homodimer, serves as an intermediary (Levchenko et al., 2000), binding the ssrA element in a groove present in each of its subunits and "delivering" the ssrA-tagged substrate to ClpX, binding to the ClpX flexible N domains via its own flexible C-terminal tails (Sauer et al., 2004). Yet, notably, the ClpX ring assembly has affinity on its own for ssrA-tagged proteins (Kim et al., 2000). By contrast, another terminal recognition element, which resides in the N-terminal region of the RepA protein, is recognized only by ClpA, which serves to dissociate the homodimeric form of RepA to a DNA binding competent monomer (Hoskins et al., 2000a). Notably, the RepA element can be recognized whether placed at the N terminus or C terminus of GFP, as judged by degradation of these substrates in the presence of ClpP (Hoskins et al., 2002).

Recent studies of ClpX have suggested, based on a mutational analysis and binding of a synthetic peptide, that it may recognize ssrA-tagged proteins via a motif shared with ClpA, the tetrapeptide GYVG (Siddiqui et al., 2004), modeled from the crystal structure of the ClpX subunit monomer (Kim and Kim, 2003) to lie at the channel-facing aspect of its single AAA+ ATPase domain. Yet a related Hsp100 protein, ClpB, which exhibits protein-disaggregation activity associated with translocation of substrates through its central channel (Weibezahn et al., 2004), has similar tetrapeptides (KYRG and GYVG) modeled to lie at the cavity aspect of each of its two AAA+ ATPase domains (Lee et al., 2003), but it fails to recognize ssrA-tagged substrates. Notably, ClpA also has two AAA+ domains and two motifs identical to those in ClpB. Here, to address the question of which sequences in ClpA are involved with substrate recognition, we have taken a general approach of examining chemical crosslinks formed with ClpA by GFP-RepA and GFP-ssrA substrates bearing photocrosslinkers. The region identified by the ssrA moiety, which faces the central channel of ClpA, as well as neighboring channel-facing structures, was then characterized by mutational study.

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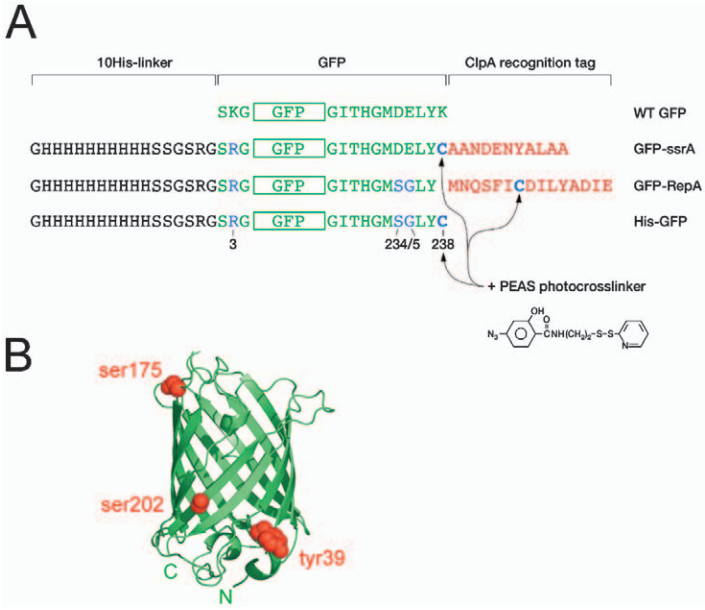

Figure 1. Photocrosslinker Placement at Tag and Body Region of ClpA Substrate Proteins GFP-ssrA and GFP-RepA(1–15)

(A) Placement of crosslinker immediately adjacent to or within C-terminal recognition tags (in red). A single accessible cysteine was substituted for lysine at the C terminus of the GFP moiety, and the photoactivatable arylazide crosslinker, PEAS (*N*-((2-pyridyldithio) ethyl)-4-azidosalicylamide), was attached. Note that amino acid substitutions were also programmed at other positions in the terminal flexible regions of GFP (numbered and shown in blue) in order to protect these sites from cleavage during postcrosslinking steps of LysC or GluC proteolytic digestion (see Figure 2A). These alterations in the terminal sequences of GFP did not affect the kinetics of unfolding and degradation of the GFP-tag fusion proteins.

(B) Placement of crosslinker on the external aspect of the GFP "body" from the GFP-ssrA and GFP-RepA(1–15) substrate protein. The three residues that are individually substituted with cysteine and PEAS labeled are indicated in red on the model of native GFP (PDB ID code 1EMA).

Results

Chemical Crosslinking Studies

Crosslinking of GFP-Tag Fusion Proteins Bearing a Photoactivatable Group to ClpA

Each of the six identical subunits of the ClpA ring is composed of three stacked domains, arranged proximal to distal in both primary and tertiary structures as N domain, AAA+ D1, and AAA+ D2. Loops at the distal surface of the D2 domains form contacts with the coaxially associating protease, ClpP. In order to map where recognition tags become bound to ClpA, we employed a crosslinking strategy, placing the bifunctional photoactivatable crosslinker, PEAS (*N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide), directly onto a cysteine residue substituted within or immediately adjacent to the tag in fusion proteins joining the stable β barrel protein GFP with either the ssrA sequence or the first 15 residues of RepA (Figure 1A). Such fusion proteins have been previously observed using fluorescence measurements to be efficiently unfolded by ClpA in the presence of ATP and also to be rapidly degraded if ClpP is present, reflecting the behavior of bone fide substrates (Weber-Ban et al., 1999; Hoskins et al., 2002). In a further set of experiments, the PEAS crosslinker was placed onto cysteine residues substituted into the solvent-exposed surface of the GFP "body" portion of these fusion proteins (Figure 1B), allowing mapping of where this portion of the substrate protein becomes localized at ClpA.

Photocrosslinking was carried out between PEAS-labeled proteins and ClpA that had been assembled into its functional form, a hexameric ring, by incubation with the nonhydrolyzable analog, ATPγS (top, Figure 2A). While this nucleotide supports assembly of ClpA subunits, it does not support active unfolding, allowing the step of substrate binding to be distinguished from a subsequent one of unfolding (Weber-Ban et al., 1999; Hoskins et al., 2000b). Because GFP-ssrA and GFP-RepA bind to ATPγS-assembled ClpA without becoming unfolded, they maintain their fluorescence, enabling detection of the substrate through subsequent steps (see Figure 2A). Because the stable GFP moiety is relatively resistant to LysC and GluC as compared to ClpA, it was possible to recover proteolytic products via nickel-affinity purification through an N-terminal His_{10} tag on intact GFP.

An SDS-PAGE analysis of a representative crosslinking and purification is shown in Figure 2B. Crosslinked species the size of GFP-tag substrate + ClpA (~115 kDa) were observed both by fluorescence of the GFP moiety and also directly by Coomassie staining. These were only obtained when the tag was present (compare "no tag" in fluorescence and Coomassie analyses), indicating specificity of the tags in recruiting the respective substrates to ClpA. While multiple discrete crosslinked products were observed (right-hand lanes), these are likely to be multiple folded conformers of the crosslinked species as opposed to products of different masses because when the crosslinked products were examined by HPLC/MS, only one mass was observed (not shown). The step of nickel-affinity purification removed a large percentage of the ClpA subunits that had failed to become crosslinked, theoretically >80% because the stoichiometry of binding of the substrate appears to be one GFP-ssrA per hexamer (Figure 2B; Piszczek et al., 2005). The further step of MonoS chromatography effectively separated noncrosslinked GFP-tag molecules from those crosslinked to ClpA. After proteolysis and affinity capture on nickel matrix, HPLC/MS was carried out.

Crosslinks Observed from the Tag Region

ssrA. When His_{10}-GFP-ssrA-crosslinked ClpA subunits were digested with LysC and recovered by Ni affinity, only large GFP adducts were recovered by HPLC/MS, including a species whose size was consistent with His_{10}-GFP-ssrA-PEAS plus a fragment of ClpA corre-

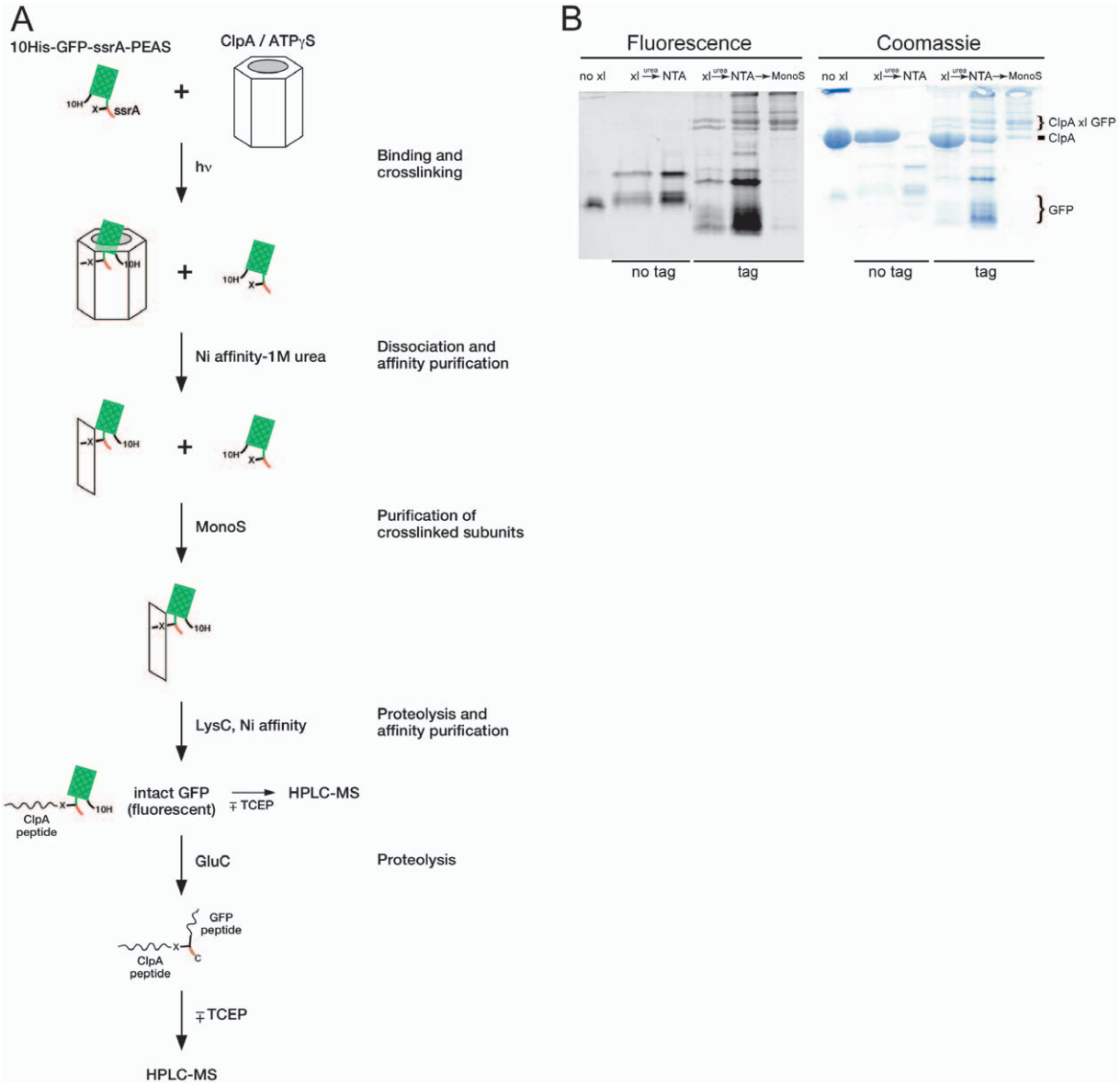

Figure 2. Scheme for Photocrosslinking the PEAS-Labeled His_{10}-GFP-ssrA Substrate to ClpA, Purifying Crosslinked ClpA Subunit, and Identifying by Proteolysis-HPLC/MS the Crosslinked Site on ClpA

(A) PEAS crosslinker, represented by an x, was attached to a cysteine substituted immediately proximal to the C-terminal ssrA tag, designated here as a red line (see also Figure 1A). Crosslinked ClpA subunit was recovered by nickel-affinity and MonoS chromatography. LysC proteolysis was then carried out, followed by nickel-affinity chromatography to recover intact GFP-ssrA crosslinked to a proteolytic fragment of ClpA. An aliquot was subjected to HPLC/MS analysis. Because the GFP moiety has an accessible glutamate, further proteolysis by GluC produced a crosslinker-bearing proteolytic fragment cleaved not only in the ClpA portion but also in the GFP portion. Aliquots were analyzed by HPLC/MS both without and with reduction by TCEP.

(B) Representative gel analysis of crosslinking (xl) and purification steps, detected by fluorescence and protein staining (Coomassie). Without photolysis (no xl) or in the absence of the recognition tag on GFP (His-GFP in Figure 1A), no crosslinked products were observed (left three lanes in each panel, "no tag"). In its presence (right three lanes, "tag"), multiple crosslinked species were observed, presumed to represent multiple conformations of a single species identified by mass spectrometry (see text). Note that to maintain the crosslinks and the GFP fluorescence, no reductant was present in the SDS sample buffer, and the samples were not heated before loading. GFP fluorescence on the gel was detected by a Storm Phosphorimager in the blue fluorescence mode.

sponding to aa 511–555 (not shown). When HPLC/MS was carried out on this sample after TCEP reduction (which separates ClpA-PEAS from GFP-ssrA; see Figure 1), a peptide was indeed observed with the size of ClpA LysC peptide 511–555 coupled with PEAS (Figure 3A). No other peptide corresponding to a LysC peptide of ClpA coupled to PEAS was recovered.

To confirm this assignment, the purified (nonreduced) LysC products were digested with GluC, which would be predicted to cleave the LysC 511–555 peptide after

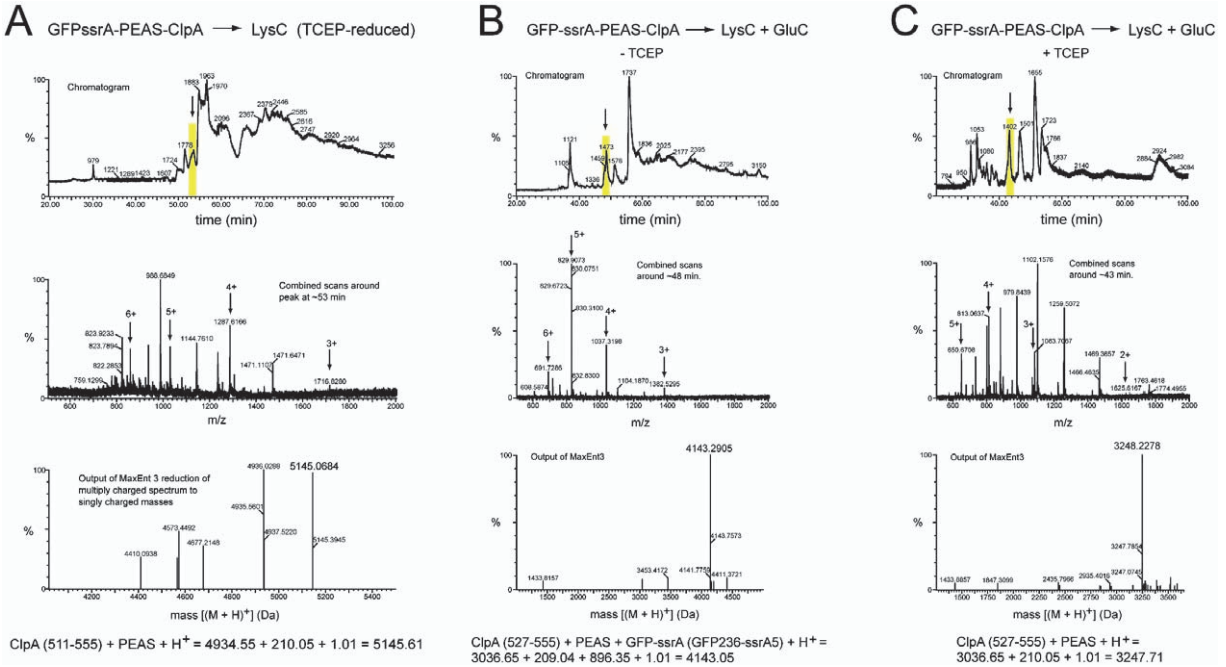

Figure 3. HPLC/MS Identifying a Crosslinked Peptide from the D2 Domain of ClpA

HPLC/MS (A) after LysC digestion of the purified GFP-ssrA-PEAS-ClpA photocrosslinked species and (B and C) after a further GluC digestion of the GFP-ssrA-PEAS-ClpA peptides purified after LysC digestion is shown.

(A) GFP-ssrA and ClpA were photocrosslinked, and the products were purified, digested with LysC, and recovered as in Figure 2 and Experimental Procedures. An aliquot was incubated with 2 mM TCEP (to reverse the PEAS crosslink) and subjected to HPLC/MS to identify the peptides. One of these (5145.0684 Da) from the indicated chromatogram peak (top panel, yellow highlight and arrow) corresponds closely to the molecular mass of a LysC peptide of ClpA (aa 511–555; 4934.55 Da) plus PEAS (210.05 Da) and a proton (total predicted mass: 5145.61 Da).

(B) The affinity-purified products of the LysC digestion in (A) were digested with GluC as in Experimental Procedures. An aliquot was subjected to HPLC/MS without TCEP reduction. One peptide (4143.2905 Da) from the indicated chromatogram peak (yellow) corresponds to a LysC-GluC double-digestion product of ClpA (aa 527–555; 3036.65 Da) combined with PEAS (209.04 Da), a peptide from GFP-ssrA that contains the crosslink-modified cysteine (GFP236-ssrA5; 896.35 Da), and a proton (total mass: 4143.05 Da). Note that this peptide contains a disulfide bond between the Cys in the GFP-ssrA peptide and the PEAS, so the mass of each is reduced by one proton.

(C) An aliquot of the GluC-digested material in (B) was subjected to HPLC/MS after reduction with 2 mM TCEP. The peptide with a mass of 3248.2278 Da corresponds to ClpA (527–555) plus PEAS and a proton (total mass: 3247.71 Da). The GFP-ssrA fragment has been released by reduction, and the PEAS sulfhydryl group is protonated.

position 526. HPLC/MS without TCEP reduction detected a peptide consistent with the predicted GluC-LysC fragment (aa 527–555) coupled via PEAS to a predicted fragment bearing the C-terminal residues of GFP (after E235) and the N-terminal five residues of ssrA (see Figures 1A and 3B). After TCEP reduction, HPLC/MS now directly revealed a peptide whose mass was consistent with ClpA(527–555) plus PEAS (Figure 3C). The released fragment of GFP-ssrA (GFP236 through ssrA5) was also detected by MS, migrating in the HPLC peak at 32.7 min. Because of the coelution of the ClpA-PEAS fragment with several larger peptides derived from GFP-ssrA, it was not possible to analyze this fragment further to determine the site of PEAS attachment.

The ClpA segment (aa 527–555) identified by this approach maps to the second AAA+ ATPase domain, D2, lying between its Walker A and B motifs, and corresponds in the crystal structure of the ClpA monomer (Guo et al., 2002) to a loop followed by an α helix (Figure 4A). The position of this region relative to an intact ring could be modeled by homology to mouse p97, a related hexameric ring assembly also containing two stacked AAA+ ATPase domains, for which an X-ray structure of an intact ring has been determined (DeLaBarre and Brunger, 2003). As shown in Figures 4A and 4B, there is substantial secondary and tertiary structural similarity of ClpA and p97 through the D2 regions of their subunits between the Walker motifs. The loop present in the crosslinked peptide of ClpA (lower portion of heavy green segment in Figure 4B, centering on aa 531) corresponds in p97 to a loop facing its central channel (Figure 4C), directed toward its 6-fold symmetry axis. The loop in ClpA, however, is almost three times longer than that in p97 (Figure 4B).

RepA. In the case of crosslinking a GFP-RepA construct, a different result was obtained. Here, a large peptide detected after LysC digestion and TCEP reduction corresponded to the N domain fragment (aa 1–144) of ClpA plus PEAS (see Figure S1A in the Supplemental Data available with this article online). A fragment corresponding to the one recovered with GFP-ssrA was not present. After further proteolysis with GluC, the large peptide was no longer observed, but several peptides were detected that were consistent with LysC-

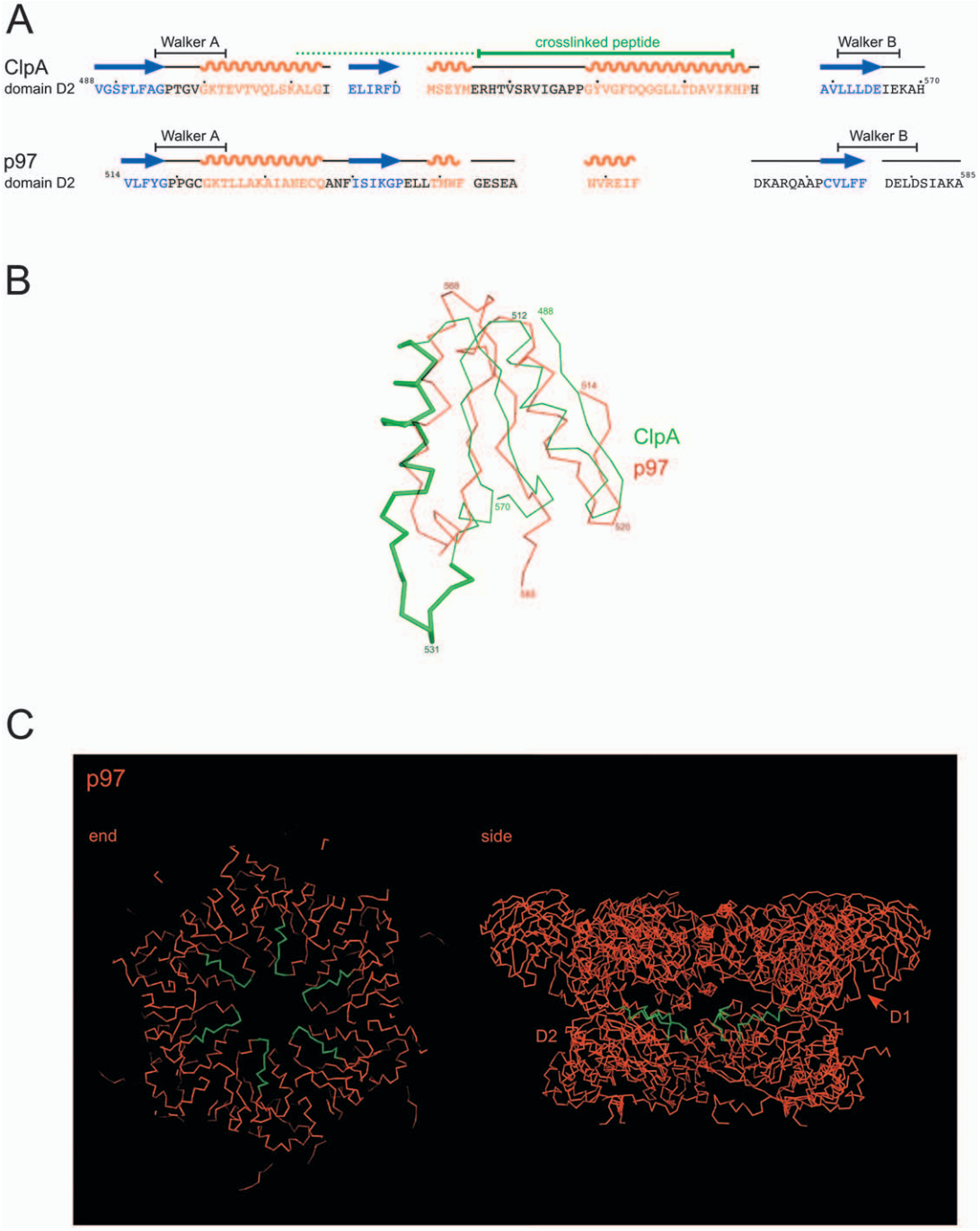

Figure 4. Localization of the ClpA Peptide Crosslinked by GFP-ssrA-PEAS

(A) and (B) show the location of the peptide in the ClpA subunit monomer, as determined from its X-ray structure. (C) shows the position of the analogous loop and α helix in the X-ray structure of the intact hexamer of the related chaperone p97.

(A and B) Comparison of primary and secondary structures of ClpA and p97 in the region of their AAA+ D2 domains between the Walker motifs. The sequences are aligned according to shared secondary structural features as shown in (B), where the X-ray structure of the ClpA monomer (PDB ID code 1KSF; Guo et al., 2002) and that of the subunit of p97 (PDB code 1OZ4; DeLaBarre and Brunger, 2003) are superposed for the D2 region between the Walker motifs. The secondary structures align closely, except in the region of the crosslinked peptide (overlined in solid green in [A], heavy green line in [B]), where there is both a larger loop in ClpA (aa 526–538) and a longer α-helical segment (aa 539–557). In (A), the additional N-terminal sequences of the larger crosslinked peptide (aa 511–555) identified by the initial LysC digestion (prior to GluC treatment) are denoted by a dotted green line.

(C) Putative quaternary structural localization of the crosslinked ClpA peptide, as indicated from position of the related segment of p97, colored green in the X-ray structure of the p97 hexamer. Note that the loop portion of this segment faces the central channel at the proximal aspect of the D2 domain.

GluC peptides from the N domain of ClpA coupled to PEAS. These included one predicted to contain the N terminus (aa 1–5) of ClpA (not shown) and one with ClpA residues 29–126 (Figure S1B). Because the N domains are flexibly tethered to the D1 ATPase domain (Ishikawa et al., 2004), it is not possible to judge from these crosslinks whether the RepA tag binds at an axial or outside aspect of the domain.

Crosslinking via the GFP Body of GFP-ssrA and GFP-RepA

When cysteine residues substituted into the external surface of the GFP moiety of GFP-ssrA or GFP-RepA (see Figure 1B) were modified with PEAS and crosslinked to ClpA, different crosslinking efficiencies were observed from different positions (data not shown), but the S202C substitution gave interpretable results with both tags. For both ssrA and RepA S202C constructs, crosslinks were detected with peptides corresponding to LysC-GluC fragments of the N domain, once again including the N-terminal (aa 1–5) fragment (Figure S2). As a further measurement of the position of the GFP body relative to the N domains, a fluorescence resonance energy transfer (FRET) experiment was carried out (Figure S3). The GFP fluorophore was the donor, and tetramethylrhodamine (TMR) was the acceptor, placed on cysteines substituted at either of two different positions on the N domains of ClpA, one proximal to the root of the domain, C97, and the other at a distal point, C72. Upon binding either GFP-ssrA or GFP-RepA, both TMR-labeled ClpAs produced significant FRET, detected by a decrease of donor fluorescence lifetime. In agreement with the physical crosslinking data, these data place the GFP body portion of the substrate within or adjacent to the N domains, presumably in an axial position inside of them.

Structure-Function Studies

Structure-Function Analysis of the ssrA-Crosslinked Segment of D2—Residues in a Channel-Facing Loop Are Critical to Binding

To evaluate whether the channel-facing segment of D2 that had been photocrosslinked by the ssrA region of GFP-ssrA was directly involved in recognizing the ssrA element, a structure-function analysis was carried out. A series of single amino acid substitutions was produced along the length of the segment, involving both a loop portion that projects into the central channel in the homologous p97 (Figure 4C) and an α helix portion (see Figure 5A). Both alanine substitutions and more drastic substitutions to acidic character were made, the latter potentially able to produce charge-charge repulsion with the acidic (D_4–E_5) region of the ssrA element (see Figure 1A).

After expression and purification, all of the substituted complexes assembled as hexamers in ATP. Strikingly, none of the five substitutions in the α-helical region affected GFP-ssrA degradation in the presence of ATP and ClpP (Table S1), but a number in the loop portion did. The most severe substitutions, L533D and G535D, completely blocked GFP-ssrA degradation (Figure 5B). Interestingly, the L533D substitution significantly inhibited ATPase activity, to ~20% of wild-type, whereas the G535D substitution had no effect on ATP turnover. Degradation was also significantly slowed by the substitutions V530D, I534C, and I534D, while R532A and I534A were without effect (Figure 5B and Table S1). In this less severe group, V530D exhibited strong reduction of ATPase activity, while the other mutants were unaffected.

To assess whether these degradation-defective substitutions were affecting recognition of ssrA by ClpA, as opposed to a later step of translocation of already-recognized substrates, we measured substrate binding by two different means. One employed the original crosslinking reaction with PEAS-labeled GFP-ssrA that had identified the D2 loop-containing region (Figure 5C). The other measured binding of fluorescein-labeled λ repressor(1–93)-ssrA (λR-ssrA) by fluorescence-anisotropy changes, as employed in earlier studies (Reid et al., 2001; Figure 5D). For both assays, the results were the same. The loop mutants that had been defective in degradation were defective both in crosslinking to PEAS-GFP-ssrA and in binding λR-ssrA as compared with wild-type ClpA (e.g., L533D and I534C; see Table S1).

Y540A Substitution in D2 Allows ssrA-Substrate Binding but Blocks Protein Translocation/Degradation

In contrast with defective ssrA-substrate binding by the D2 loop mutants, a substitution just beyond the loop, Y540A, lying in the proximal region of the α helix in a conserved GYVG motif, could bind ssrA substrates but could not degrade them (Figure 5). In particular, it was unable to degrade GFP-ssrA in the presence of ATP and ClpP (Figure 5B), but it could nonetheless be crosslinked by the GFP-ssrA-PEAS substrate (Figure 5C) and bound by λR-ssrA (Figure 5D), although somewhat less extensively than wild-type. This implies that, while binding of GFP-ssrA to this mutant can occur, subsequent steps of unfolding/translocation and degradation are blocked. Given the putative location of Y540 at the region of insertion of the loop into the channel wall of ClpA, we surmise that this residue could function in movement of the loop in association with translocation and degradation (see Discussion).

Involvement of the D2 Loop in Translocation/Degradation of GFP-RepA

The D2 loop mutants as well as Y540A were also tested for ability to bind GFP-RepA and to mediate its degradation in the presence of ATP and ClpP. Using the PEAS-labeled GFP-RepA substrate (Figure 1A) in a crosslinking reaction with the mutants, we observed in all cases a similar extent of crosslinking compared to that with wild-type ClpA (Figure 5E). This is consistent with the results of the foregoing crosslinking studies that mapped recognition of the RepA tag to the N domains, distant from the D2 loop. Despite such normal binding, the D2 loop mutants were unable to degrade GFP-RepA in the presence of ATP and ClpP (Figure 5F). This suggests that the same D2 residues involved with ssrA binding are also involved with binding, unfolding, and translocation of GFP-RepA after ATP-dependent release from its site of initial binding to the N domains in ATPγS.

Residues in Channel-Facing Loops of the D1 ATPase Are Also Critical to ssrA Binding and Translocation/Degradation of GFP-RepA

Inspecting the primary structure of the D2 loop segment that had been functionally analyzed (Figure S4), we were struck on one hand by the lack of a corre-

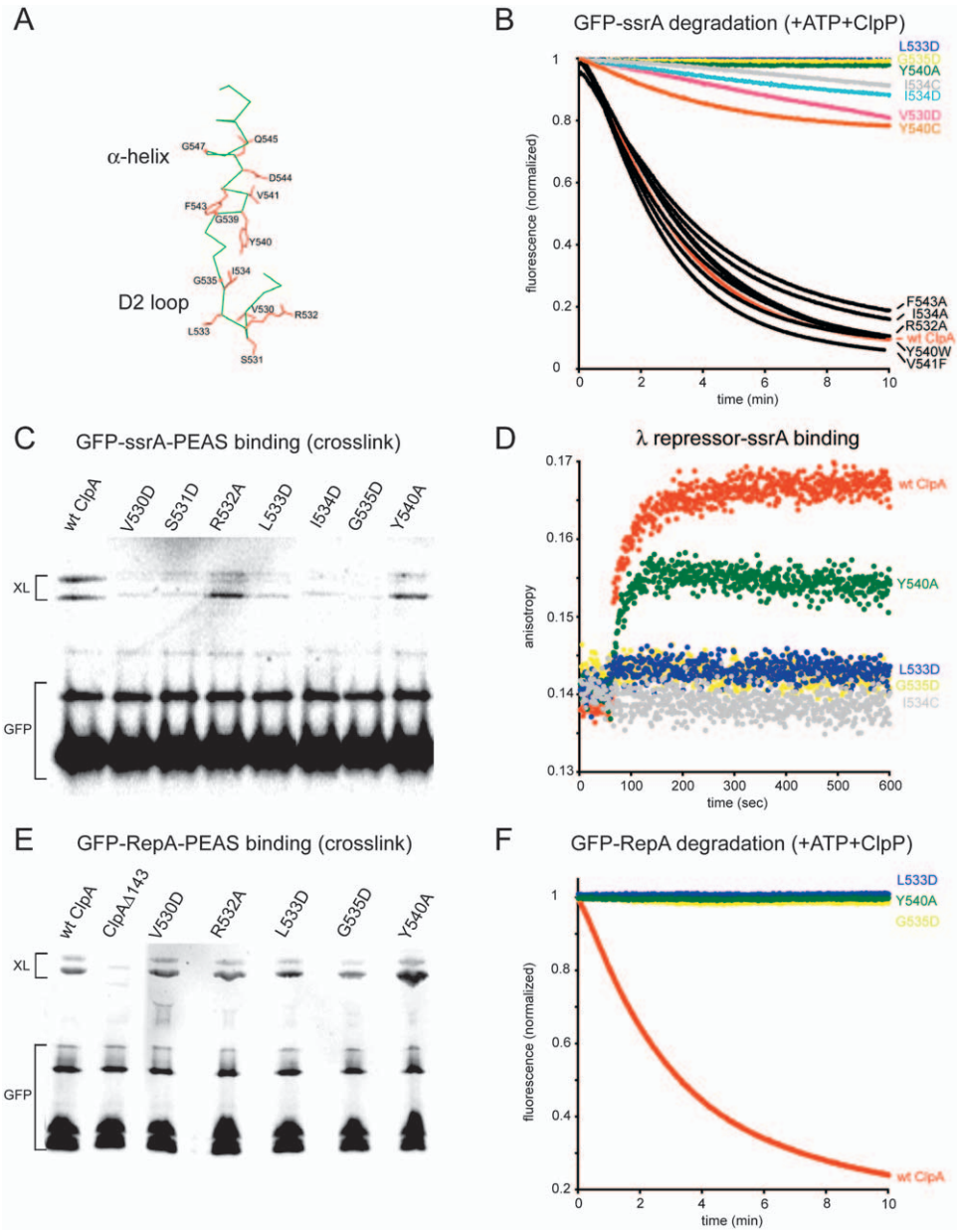

Figure 5. Structure-Function Analysis of the D2 Loop Region Identified by Crosslinking

(A) View of the D2 segment crosslinked by GFP-ssrA, with the main chain in green and side chains that have been mutated in red and numbered. This view is from the ClpA channel, as modeled on the structure of p97 (see Experimental Procedures).

(B) Degradation of GFP-ssrA by wild-type ClpA and the ClpA D2 mutants in the presence of ClpP and ATP. The reaction was followed by loss of GFP fluorescence, as detailed in Experimental Procedures. A number of mutants (black traces) were as active as wild-type ClpA (red trace). A few were completely inactive (L533D, G535D, and Y540A; dark blue, yellow, and green traces, respectively); several others (V530D, I534C, I534D, and Y540C) had reduced but detectable activity.

(C) Binding of GFP-ssrA-PEAS to ClpA and several ClpA D2 mutants determined by photocrosslinking. Photocrosslinking was carried out as in Experimental Procedures and Figure 2, except on a smaller scale. Aliquots were electrophoresed on an SDS gel without reduction or heating to retain both the disulfide crosslink and GFP fluorescence. GFP fluorescence was detected by Phosphorimager. Crosslinked products are indicated by "XL" and noncrosslinked GFP-ssrA by "GFP." Some mutants show strong crosslinking, indicative of binding, including Y540A, even though it failed to degrade GFP-ssrA (see [B]); others, such as G535D, show no crosslinking, consistent with their inability to degrade GFP-ssrA. In comparison with the wild-type lane, the major crosslinked species with the mutants was the faster-migrating one, but the basis for this difference is not known.

(D) Binding of another ssrA-tagged ClpA substrate, λR-ssrA, to selected mutant ClpAs. The change in anisotropy of fluorescein-maleimide-labeled λR-ssrA upon ATPγS addition (at 60 s) was monitored as in Experimental Procedures. Color coding is as in (B). This substrate binds to mutant Y540A, although less well than it does to wild-type ClpA.

(E) Binding of GFP-RepA to wild-type ClpA and selected D2 mutants. GFP-RepA-PEAS was incubated with ClpA and the indicated mutants and photocrosslinked as in (C). All of the mutants in D2 show strong crosslinks. Only ClpAΔ143 (missing the N domain) fails to crosslink, as expected because the N domain is required for binding the RepA tag. Selected lanes from the same gel are shown.

(F) Degradation of GFP-RepA by wild-type ClpA and selected ClpA D2 mutants in the presence of ClpP and ATP. GFP-RepA was incubated with ClpA or the indicated D2 mutants, ClpP, and ATP, and its fluorescence was followed as in (B). Color coding is as in (B). Note that none of the mutants can degrade GFP-RepA, even though all of them can crosslink this substrate (see [E]).

sponding structure in ClpX, which, like ClpA, acts on ssrA-tagged substrates, and on the other by the presence of strong homology with ClpB, which does not recognize ssrA-tagged proteins (M. Zolkiewski, J.H., and A.L.H., unpublished data). Meanwhile, both ClpX and ClpB D2 share a GYVG motif with the D2 of ClpA. Such structural and functional disparities with related components prompted consideration that the ClpA D2 loop sequence might not function alone in mediating ssrA-substrate binding. Therefore, we carried out a further functional analysis of neighboring structures facing the central channel at the level of the proximal ATPase domain (D1). Identical measurements of ssrA-substrate binding and unfolding/translocation/degradation were carried out and revealed that single-residue substitutions in many residues in both a D1 loop with homology to that of D2 (termed loop 1) and a neighboring loop (loop 2) abolished ssrA binding (Figures 6A–6D and Table S1). By contrast, substitutions in an underlying α helix in D1 were without effect (Figure 6A).

The mutants were also tested with GFP-RepA, and, as expected, the mutations blocking ssrA-substrate binding did not affect GFP-RepA binding (Figure 6E). On the other hand, these mutants, like those of D2, abolished GFP-RepA degradation (Figure 6F). Thus, these D1 residues likely participate in subsequent binding and/or unfolding of GFP-RepA initially bound by the N domains.

Substitutions in Loops of Both D1 and D2 Block ClpA's Ability to Bind Unfolded Proteins Lacking a Tag

It seemed unclear whether the identified loop residues were involved exclusively with binding the ssrA tag or whether these elements could also bind additional sequences in recognized substrates, possibly as they begin to unfold and translocate through the central channel. The question of involvement in binding segments of unfolding polypeptide was addressed by taking advantage of previous observations that ClpA has significant affinity for unfolded proteins lacking tag sequences. Two such proteins have been studied: λR (without a tag), which unfolds dynamically in solution (Huang and Oas, 1995), and GFP diluted from denaturant (Hoskins et al., 2000b). Both proteins were able to bind to ClpA and be degraded by ClpAP. Here, these same substrates were tested with the D1 and D2 loop mutants. In an assay of fluorescence anisotropy of fluorescein-labeled λR, we observed that substitutions that had blocked ssrA binding also blocked binding of λR (Figure 7A). The substitution, Y540A, that had been able to bind but not translocate GFP-ssrA also behaved concordantly, able to at least partially bind λR (Figure 7A) but unable to efficiently degrade it when compared with wild-type ClpA (Figure 7B), although here the reductions in apparent binding and degradation were of similar magnitude. Furthermore, in an assay of binding acid-unfolded GFP measuring loss of recovery of GFP fluorescence as a function of binding to ClpA (unbound GFP spontaneously refolds to the fluorescent native state), the pattern of behavior of the mutants once again paralleled that toward the ssrA substrates (Figure 7C). We thus conclude that the loop residues critical to binding ssrA-tagged substrates are likewise involved with binding proximal segments of nonnative polypeptide entering the central channel during ClpA-mediated unfolding and translocation.

Discussion

Cooperation of Channel-Facing Loops in Substrate Binding by ClpA

The mutational studies here indicate a role for three loops in the central channel of ClpA in binding both the ssrA tag element and segments of unfolded polypeptide. Two of these loops are situated at the level of the D1 domain, while a third, which was chemically crosslinked by GFP-ssrA and also tested functionally, is localized to the D2 domain. We propose that binding involves the participation of these three loops in concert because substitutions in any of the three produced, in many cases, a complete block of binding. This seems incompatible with a sequential transfer of substrate from one loop(s) to another, as, for example, in a "bucket brigade" model of passing of substrate from a D1 binding site to one in D2. In such a model, for example, one would have expected that binding could still occur through D1 in the setting of D2 loop substitutions.

Yet mutants defective in binding ssrA-tagged proteins and tagless unfolded proteins were also defective in GFP-RepA degradation (in the presence of ATP and ClpP). Because the loop mutant versions of ClpA remained able to bind GFP-RepA (presumably via the N domains, as in the case of wild-type ClpA), this implies that GFP-RepA ultimately takes the same path through ClpA as ssrA substrates, requiring binding and translocation by the same channel-facing loops in D1 and D2. It also implies that transfer does in fact occur from the N domains to these loops. The nature of the ATP-mediated action that recruits the RepA tag from the N domains remains to be resolved. For example, whether the loops from D1 can reach up to contact the substrate or whether the N domains bend down to deliver the substrate to the channel is unknown.

Nature of ssrA Binding

The crosslinking of the ssrA region of GFP-ssrA to a loop as far down the ClpA channel as D2 was something of a surprise, but the functional results and consideration of the geometry of substrate binding and of ClpA itself lend support to such a direct physical contact. We infer that GFP-ssrA occupies a topology while bound at ClpA that positions the folded GFP moiety axially inside the N domains, as indicated by both chemical crosslinking from the GFP body (Figure S2) and a FRET study (Figure S3), and in this position can extend the flexible C-terminal segment down the channel past the face of D1 (Figure 8). The ssrA tag is relatively unstructured while bound in a cocrystal to the delivery protein SspB (Levchenko et al., 2003; Song and Eck, 2003), and, if it occupies an extended conformation in solution and when interacting with ClpA, it could extend as far as 35 Å. There are another eight to ten residues at the C terminus of GFP that are disordered, potentially adding an additional 25 Å of length. From a working model of ClpA derived from the crystal structure of the subunit monomer and from EM studies (Ishikawa et al., 2004), we estimate the height of the D1

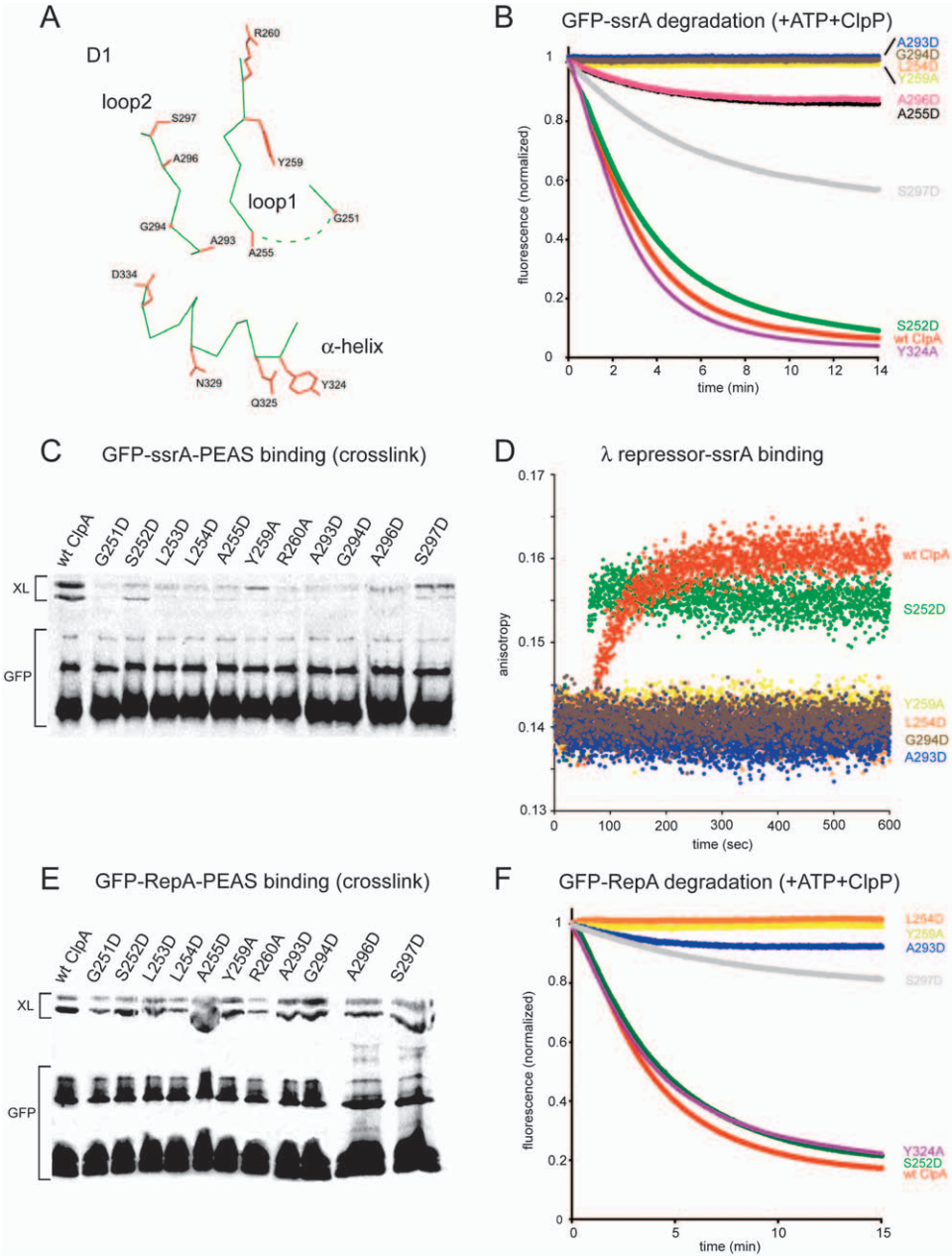

Figure 6. Structure-Function Analysis of the D1 Region of the Central Channel

(A) View of two loops and an underlying α helix in the D1 channel-facing region, with the main chain in green and side chains that have been mutated in red and numbered. View is from the ClpA channel, as modeled from the structure of p97 (see Experimental Procedures).

(B) Degradation of GFP-ssrA by wild-type ClpA and ClpA D1 mutants in the presence of ClpP and ATP, carried out as in Figure 5B.

(C) Binding of GFP-ssrA-PEAS to ClpA and several ClpA D1 mutants determined by photocrosslinking. Photocrosslinking was carried out and analyzed as in Figure 5C. Crosslinked products are indicated by "XL" and noncrosslinked GFP-ssrA by "GFP."

(D) Binding of another ssrA-tagged ClpA substrate, λR-ssrA, to selected mutant ClpAs. The change in anisotropy of fluorescein-labeled λR-ssrA upon ATPγS addition was monitored as in Experimental Procedures. Color coding is as in (B).

(E) Binding of GFP-RepA to wild-type ClpA and selected D1 mutants. GFP-RepA-PEAS was incubated with ClpA and the indicated mutants and photocrosslinked as in (C). All of the selected mutants show strong crosslinks.

(F) Degradation of GFP-RepA by wild-type ClpA and selected ClpA D1 mutants in the presence of ClpP and ATP. GFP-RepA was incubated with ClpA or the indicated D1 mutants, ClpP, and ATP, and its fluorescence was followed as in (B). Color coding is as in (B). Note that some of the mutants (e.g., L254D, Y259A) cannot degrade GFP-RepA, even though all of them bind this substrate (see [E]).

domain as 40 Å. Thus, the combined lengths of the unstructured C terminus of GFP and the ssrA tag would allow the ssrA element to extend to a level where the D2 loop could directly contact it, particularly if the loop can assume a proximal position within the central channel. Of course, an allosteric effect of one loop on the

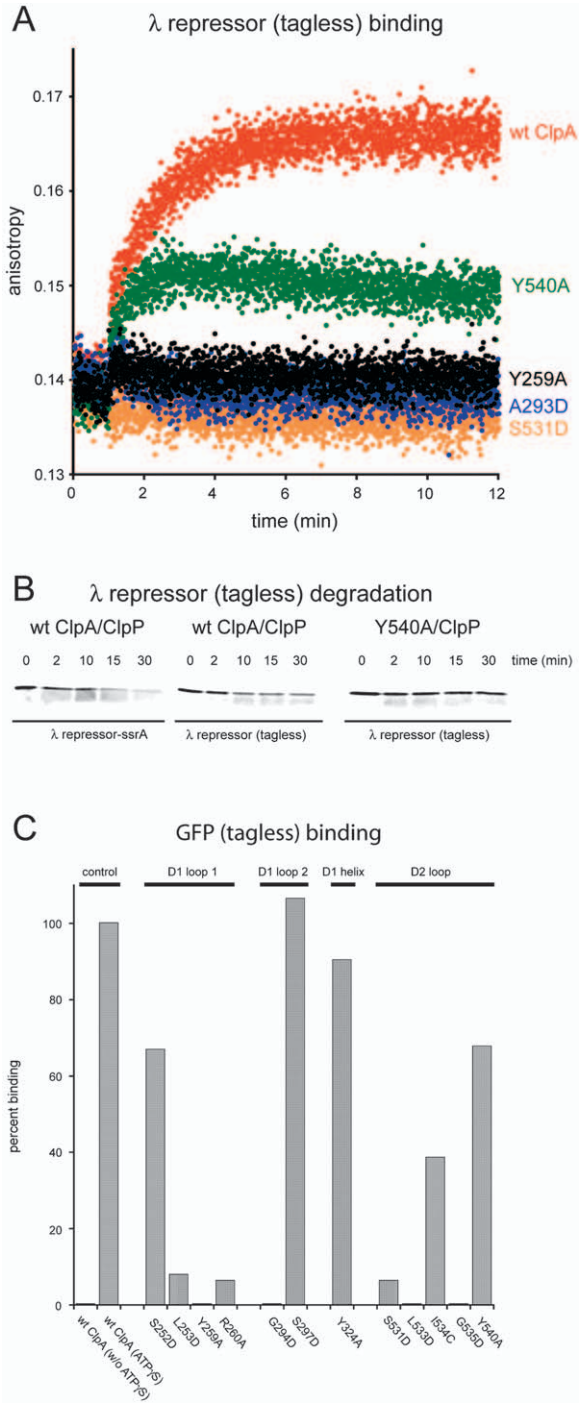

Figure 7. Binding and Degradation of Two Unfolded Substrates, λR and GFP, by ClpA and Mutants in the Loop Segments

(A) Binding of λR (without a tag) to representative mutant ClpAs. Fluorescein-labeled λR was incubated with wild-type ClpA (red trace) or representative mutants from D1 and D2 regions, and binding was followed by the change in anisotropy upon addition of ATPγS at 100 s. Y540A (green trace) binds λR, although with lower efficiency than wild-type ClpA. The other mutants, defective in binding ssrA-tagged substrates, are also defective here.

(B) Degradation of λR by wild-type and Y540A ClpA in the presence of ClpP and ATP. Fluorescein-labeled λR was incubated with the indicated ClpA, ClpP, and ATP as in Experimental Procedures. Aliquots were removed at the indicated times and electrophoresed on an SDS-polyacrylamide gel. Intact labeled λR was detected by the blue fluorescence channel of a Storm Phosphorimager. Degradation of λR-ssrA by wild-type ClpA/ClpP (left panel) is included for comparison.

(C) Unfolded-GFP binding by wild-type ClpA and various mutants. Inhibition of recovery of GFP fluorescence upon dilution from denaturant was used as a measure of binding to ClpA, as in Supplemental Experimental Procedures. No binding (e.g., wild-type ClpA without ATPγS) corresponds to maximum recovery of fluorescence, while 100% binding (wild-type ClpA with ATPγS) is minimum recovery (about 65% of the starting fluorescence). Mutants in all three loops that failed to bind tagged substrates also fail to bind unfolded GFP. Those that showed partial binding of tagged substrates, such as Y540A, also show partial binding here.

other cannot be excluded based on the present studies. We note, however, that many of the loop substitutions made here had no remote effects on hexameric assembly or ATPase activity (see Table S1).

The nature of the putative binding site formed by these three loops remains unclear. Perhaps a structure resembling the shallow groove where ssrA binds in SspB (Levchenko et al., 2003; Song and Eck, 2003) can be formed by interaction of the three loops in ClpA, albeit that a distinct mechanism of binding may also be involved. On the part of the ssrA tag itself, considering that the ClpA loops also participate in recognition of unfolded polypeptides devoid of tag elements, the question must be raised as to whether it is simply a segment of "generic" unfolded polypeptide bearing a relatively apolar sequence. Consistent with this, if the ssrA sequence, AANDENYALAA, in GFP-ssrA was substituted at positions 1, 2, 8, or 9 with aspartate, binding was reduced, while substitutions of the polar residues with alanine were without effect (Flynn et al., 2001). As concerns recognition of proximal segments of a substrate protein, once unfolding commences, such elements would have access to the central channel, access that would be sterically excluded while in native globular structure (see Figure 8). In addition, as such elements enter the channel, they would be present at very high local concentration, potentially overcoming any sequence specificity in the affinity of the channel-facing loops. Whether such binding favors, for example, hydrophobic side chains of proximal segments or involves main-chain hydrogen bonding remains to be resolved.

The cooperation of loops observed here in binding substrate proteins may also be relevant to protein binding and disaggregation mediated by the related chaperone, ClpB, which also has two ATPase domains and channel-facing loops, even though this chaperone does not recognize ssrA-tagged proteins. A recent study has shown, for example, that the D2 channel-facing region of ClpB is involved with protein disaggregation (Weibezahn et al., 2004), and an earlier study from the same group presented evidence that a loop in the D1 region might be involved (Schlieker et al., 2004), although the mutational effects at D1 were small relative to those observed at D2. Nevertheless, it may be that loops in the channel of ClpB cooperate similarly to those in ClpA in recognizing proteins introduced into this chaperone by assisting action of Hsp70 during protein disaggregation.

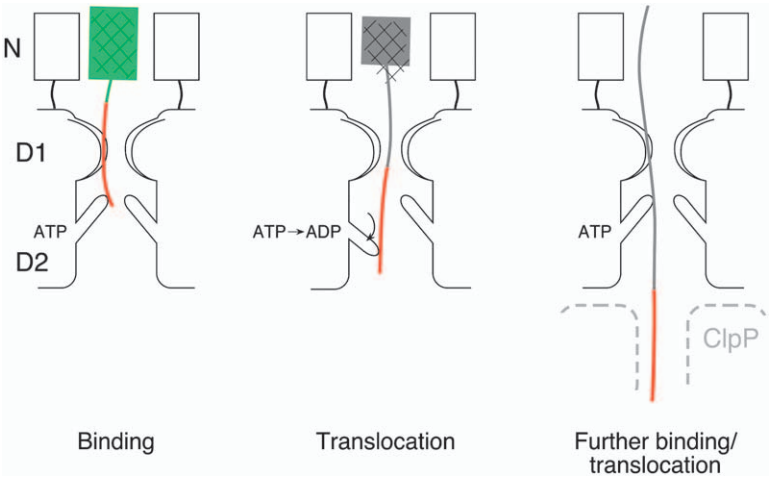

Figure 8. Model for Binding, Translocation, and Unfolding of GFP-ssrA by ClpA

Binding (left panel) requires interaction of the C-terminal ssrA tag element (red) with three loops lying in the central channel, two at the level of the D1 ATPase, and one at the level of the D2 ATPase. As illustrated, interaction is occurring with loops in one subunit, but other arrangements are not excluded. The GFP moiety (green hatched rectangle) is shown in an axial position at the level of the N domains.

Translocation (middle panel) is proposed to be triggered by ATP hydrolysis by the D2 ATPase, driving the D2 loop distally and exerting mechanical force on the ssrA tag and the C-terminal segment of GFP, acting to commence unfolding of GFP (see Discussion).

Further binding/translocation (right panel) can occur following restoration of the D2 loop to its original position upon nucleotide exchange. The possibility of binding of proximal segments of polypeptide chain by the channel loops is suggested by the affinity of ClpA for nonnative proteins lacking a tag element (Hoskins et al., 2000b) and by the loss of binding and translocation of such substrates by the same substitution mutants that adversely affect ssrA-substrate binding and translocation.

Substrate Translocation—A Proposal

We propose that the D2 loop of ClpA mediates protein unfolding by moving, along with a bound segment of substrate protein, from a proximal to a distal position inside the central channel in association with ATP hydrolysis in the D2 nucleotide pocket, effectively exerting mechanical pulling on bound substrate that unfolds it in association with its translocation (Figure 8). This proposal is based on a number of observations: location of the D2 loop inside the central channel, lying in the pathway that polypeptides take through ClpA; involvement demonstrated here in substrate-protein binding; connection to the much more active ATPase domain of ClpA, the D2 ATPase (Singh and Maurizi, 1994); observation here of a mutant in D2 that can bind but not translocate substrate polypeptide; and precedents for such loop movement inside hexameric nucleic-acid-translocating ring structures and, very recently, inside p97 (DeLaBarre and Brunger, 2005).

The functional studies here support this model by showing on one hand that the D2 loop is involved in polypeptide binding and on the other that a mutant adjoining the D2 loop can support substrate binding but is selectively defective for the subsequent steps of unfolding, translocation, and degradation (Figure 5). In particular, the ClpA mutant Y540A was able to bind ssrA substrates and also two unfolded polypeptides devoid of tag sequences, but it was impaired in their degradation in the presence of ATP and ClpP (despite normal turnover of ATP). This indicates that the coupled steps of unfolding and translocation were affected. Whether Y540A itself lies in a physical "hinge" point for the loop or, rather, lies in a pathway of transduction of ATP hydrolysis into putative loop movement remains unclear.

The size of the D2 loop, direction of polypeptide translocation, and nature of polypeptide unfolding are consistent with a model of D2 loop-mediated translocation. The D2 loop is 13 residues in size and, in modeled structures, is able to move well proximal in the central channel, up to the level of the D1 domain, but is also able, with putative distal movement, to reach well toward the distal outlet of ClpA. Consistent with such directional movement is the direction of movement of substrate proteins, with the ssrA-tagged end of substrates the first to enter the distally localized ClpP proteolytic chamber during ClpAP-mediated degradation (Reid et al., 2001). Consistent with a proposed model involving force exerted from a single point, i.e., by the D2 loop, experiments from the Matouschek and Sauer groups with ClpA and ClpX have suggested that it is the degree of local structure adjoining the tag element that determines the rate of unfolding, based on comparing rates of unfolding of substrates tagged in a variety of structural contexts. This has been interpreted to indicate that it is a local pulling force at one end of substrate proteins that appears to mediate unfolding (Lee et al., 2001; Kenniston et al., 2003).

Presumably, beyond initial movement of the ssrA-tagged end of a substrate, additional rounds of forceful movement may also be able to be exerted, at least by ClpA, because it has affinity for unfolded proteins (Figure 8). That is, more proximal portions of a substrate polypeptide chain may be bound and translocated by the same kind of movement as that exerted on the initially bound ssrA segment. This action appears to apply as well in the case of the GFP-RepA substrate, which binds initially to the N domains at the top of the ClpA cylinder. Whether GFP-RepA becomes subsequently recognized at the level of the D1 and D2 loops through the RepA tag element or through sequences proximal to its tag remains to be resolved.

The proposed model of D2 loop-mediated translocation of substrate proteins takes as precedents models recently proposed for the action of two ATP-consuming hexameric ring structures that translocate nucleic acids, the packaging motor for the bacteriophage ϕ12 and the SV40 large T antigen (Mancini et al., 2004; Gai et al., 2004). For the packaging motor, an RNA binding loop, present in each subunit facing a central channel, is involved. As captured crystallographically, the loop of any given subunit was observed to assume a different position depending on the bound nucleotide. ATP hy-

drolysis was proposed to occur sequentially in a rotary fashion around the ring of this machine, with ATP hydrolysis in any given subunit associated with a distalward displacement of the loop and an incremental movement of the bound RNA substrate. On the other hand, a similar mechanism of loop-directed translocation proposed for the SV40 large T antigen invoked a concerted ATP hydrolysis mechanism associated with a helicase action on origin DNA (Gai et al., 2004). Finally, inspection of newly published structures of p97 indicate that its D2 channel-facing loop repositions downward while going from an AMP-PNP ("ATP") bound D2 ring to an ADP bound state (DeLaBarre and Brunger, 2005). Additional structural and mechanistic studies of the ClpA D1 and D2 loops may be able to better resolve both substrate-protein binding and putative translocation movements associated with protein unfolding.

Experimental Procedures

DNA Constructs and Proteins

DNA constructs and proteins were as previously described, detailed in Supplemental Experimental Procedures.

Degradation and Binding Assays

Degradation of GFP-ssrA, GFP-RepA, and their variants with wild-type ClpA/ClpP (or the ClpA mutants) was performed essentially as described (Weber-Ban et al., 1999), detailed in Supplemental Experimental Procedures. Fluorescence measurements of binding of fluorescein-labeled λR or λR-ssrA or of GFP to ClpA are described in Supplemental Experimental Procedures.

Photocrosslinking and Sample Purification

His-tagged GFP variants reduced with TCEP were exchanged into 20 mM potassium phosphate (pH 7.5), 50 mM NaCl by PD-10 (Amersham) chromatography, then incubated with equimolar bifunctional crosslinker PEAS (Molecular Probes) dissolved in DMSO. After 1 hr at 23°C in the dark, PD-10 chromatography removed unreacted photocrosslinker and exchanged the buffer to 20 mM Tris-HCl (pH 7.5), 100 mM NaCl. Mass spectrometry verified labeling efficiency. PEAS-labeled substrate (4 μM) was added to 40 ml crosslinking buffer (300 mM NaCl, 20 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$, 10% glycerol, 0.5 mM ATPγS) containing 1.5 μM ClpA, then aliquoted into 96-well microtiter plates (75 μl/well). After photolysis (10 × 30 s at 30 s intervals; 312 nm Stratalinker), aliquots were pooled, urea was added to 1 M, and the sample was purified on a Ni^{2+}-charged HiTrap. The material was eluted with 200 mM imidazole in 1 M urea; diluted 10-fold with 20 mM Tris-HCl (pH 8.0), 1 M urea; and subjected to cation-exchange chromatography on a 5 × 50 mm MonoS column (Amersham), developed with a steep gradient (to 1 M NaCl). The fluorescence peak containing crosslinked material was collected, concentrated, and exchanged to LysC digestion buffer (100 mM Tris-HCl [pH 8.9], 1 M urea, 50 mM NaCl) on a 30 kDa Amicon Ultra-4 centrifugal concentrator (Millipore). LysC (Wako) digestion was performed for 16–18 hr at 23°C at 1:20 mass ratio (LysC:crosslinked protein). The digest was purified via Ni^{2+}-HiTrap. The recovered material was exchanged into 50 mM ammonium bicarbonate, 0.1% RapiGest (Waters) using an Ultra-4 concentrator and digested with GluC (Roche) at a 1:20 mass ratio for 16–18 hr at 23°C, then immediately subjected to HPLC/MS analysis.

HPLC/MS

HPLC/MS was carried out on a Shimadzu 10A series liquid chromatograph directly coupled to a Micromass/Waters Q-Tof1 tandem mass spectrometer with electrospray ionization, as detailed in Supplemental Experimental Procedures.

Structure Modeling

The structures of the D1 and D2 domains of the ClpA monomer (PDB ID code 1KSF; Guo et al., 2002) were superposed individually on the structure of the p97 hexamer (PDB ID code 1OZ4; DeLaBarre and Brunger, 2003) to build a model for the ClpA hexamer, using the program O (Jones et al., 1991).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, four figures, and one table and are available with this article online at http://www.cell.com/cgi/content/full/121/7/1029/DC1/.

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References

DeLaBarre, B., and Brunger, A.T. (2003). Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. Nat. Struct. Biol. *10*, 856–863.

DeLaBarre, B., and Brunger, A.T. (2005). Nucleotide dependent motion and mechanism of action of p97/VCP. J. Mol. Biol. *347*, 437–452.

Flynn, J.M., Levchenko, I., Seidel, M., Wickner, S.H., Sauer, R.T., and Baker, T.A. (2001). Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. Proc. Natl. Acad. Sci. USA *98*, 10584–10589.

Flynn, J.M., Neher, S.B., Kim, Y.I., Sauer, R.T., and Baker, T.A. (2003). Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. Mol. Cell *11*, 671–683.

Gai, D., Zhao, R., Li, D., Finkielstein, C.V., and Chen, X.S. (2004). Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. Cell *119*, 47–60.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell *94*, 615–623.

Guo, F., Maurizi, M.R., Esser, L., and Xia, D. (2002). Crystal structure of ClpA, an Hsp100 chaperone and regulator of ClpAP protease. J. Biol. Chem. *277*, 46743–46752.

Hoskins, J.R., Kim, S.-Y., and Wickner, S. (2000a). Substrate recognition by the ClpA chaperone component of ClpAP protease. J. Biol. Chem. *275*, 35361–35367.

Hoskins, J.R., Singh, S.K., Maurizi, M.R., and Wickner, S. (2000b). Protein binding and unfolding by the chaperone ClpA and degradation by the protease ClpAP. Proc. Natl. Acad. Sci. USA *97*, 8892–8897.

Hoskins, J.R., Sharma, S., Sathyanarayanam, B.K., and Wickner, S. (2001). Clp ATPases and their role in protein unfolding and degradation. Adv. Protein Chem. *59*, 413–429.

Hoskins, J.R., Yanagihara, K., Mizuuchi, K., and Wickner, S. (2002). ClpAP and ClpXP degrade proteins with tags located in the interior of the primary sequence. Proc. Natl. Acad. Sci. USA *99*, 11037–11042.

Huang, G.S., and Oas, T.G. (1995). Submillisecond folding of monomeric lambda repressor. Proc. Natl. Acad. Sci. USA *92*, 6878–6882.

Ishikawa, T., Maurizi, M.R., and Steven, A.C. (2004). The N-terminal substrate-binding domain of ClpA unfoldase is highly mobile and

extends axially from the distal surface of ClpAP protease. J. Struct. Biol. *146*, 180–188.

Jones, T.A., Zou, J.Y., Cowan, S.W., and Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A *47*, 110–119.

Keiler, K.C., Waller, P.R.H., and Sauer, R.T. (1996). Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science *271*, 990–993.

Kenniston, J.A., Baker, T.A., Fernandez, J.M., and Sauer, R.T. (2003). Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine. Cell *114*, 511–520.

Kim, D.Y., and Kim, K.K. (2003). Crystal structure of ClpX molecular chaperone from *Helicobacter pylori*. J. Biol. Chem. *278*, 50664–50670.

Kim, Y.I., Burton, R.E., Burton, B.M., Sauer, R.T., and Baker, T.A. (2000). Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. Mol. Cell *5*, 639–648.

Lee, C., Schwartz, M.P., Prakash, S., Iwakura, M., and Matouschek, A. (2001). ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. Mol. Cell *7*, 627–637.

Lee, S., Sowa, M.E., Watanabe, Y.-h., Sigler, P.B., Chiu, W., Yoshida, M., and Tsai, F.T.F. (2003). The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. Cell *115*, 229–240.

Levchenko, I., Seidel, M., Sauer, R.T., and Baker, T.A. (2000). A specificity-enhancing factor for the ClpXP degradation machine. Science *289*, 2354–2356.

Levchenko, I., Grant, R.A., Wah, D.A., Sauer, R.T., and Baker, T.A. (2003). Structure of a delivery protein for an AAA+ protease in complex with a peptide degradation tag. Mol. Cell *12*, 365–372.

Mancini, E.J., Kainov, D.E., Grimes, J.M., Tuma, R., Bamford, D.H., and Stuart, D.I. (2004). Atomic snapshots of an RNA packaging motor reveal conformational changes linking ATP hydrolysis to RNA translocation. Cell *118*, 743–755.

Piszczek, G., Rozycki, J., Singh, S.K., Ginsburg, A., and Maurizi, M.R. (2005). The molecular chaperone, ClpA, has a single high affinity peptide binding site per hexamer. J. Biol. Chem. *280*, 12221–12230.

Reid, B.G., Fenton, W.A., Horwich, A.L., and Weber-Ban, E.U. (2001). ClpA mediates directional translocation of substrate proteins into the ClpP protease. Proc. Natl. Acad. Sci. USA *98*, 3768–3772.

Sauer, R.T., Bolon, D.N., Burton, B.M., Burton, R.E., Flynn, J.M., Grant, R.A., Hersch, G.L., Joshi, S.A., Kenniston, J.A., Levchenko, I., et al. (2004). Sculpting the proteome with AAA+ proteases and disassembly machines. Cell *119*, 9–18.

Schlieker, C., Weibezahn, J., Patzelt, H., Tessarz, P., Strub, C., Zeth, K., Erbse, A., Schneider-Mergener, J., Chin, J.W., Schultz, P.G., and Bukau, B. (2004). Substrate recognition by the AAA+ chaperone ClpB. Nat. Struct. Mol. Biol. *11*, 607–615.

Siddiqui, S.M., Sauer, R.T., and Baker, T.A. (2004). Role of the protein-processing pore of ClpX, a AAA+ ATPase, in recognition and engagement of specific protein substrates. Genes Dev. *18*, 369–374.

Singh, S.K., and Maurizi, M.R. (1994). Mutational analysis demonstrates different functional roles for the two ATP-binding sites in ClpAP protease from *Escherichia coli*. J. Biol. Chem. *269*, 29537–29545.

Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell *75*, 409–418.

Song, H.K., and Eck, M.J. (2003). Structural basis of degradation signal recognition by SspB, a specificity-enhancing factor for the ClpXP proteolytic machine. Mol. Cell *12*, 75–86.

Weber-Ban, E.U., Reid, B.G., Miranker, A.D., and Horwich, A.L. (1999). Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. Nature *401*, 90–93.

Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E.U., Dougan, D.A., Tsai, F.T.F., et al. (2004). Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. Cell *119*, 653–665.

Ye, Y., Meyer, H.H., and Rapoport, T.A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature *414*, 652–656.