Substrate Recognition by the ClpA Chaperone Component of ClpAP Protease*

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ClpA, a member of the Clp/Hsp100 ATPase family, is a molecular chaperone and regulatory component of ClpAP protease. We explored the mechanism of protein recognition by ClpA using a high affinity substrate, RepA, which is activated for DNA binding by ClpA and degraded by ClpAP. By characterizing RepA derivatives with N- or C-terminal deletions, we found that the N-terminal portion of RepA is required for recognition. More precisely, RepA derivatives lacking the N-terminal 5 or 10 amino acids are degraded by ClpAP at a rate similar to full-length RepA, whereas RepA derivatives lacking 15 or 20 amino acids are degraded much more slowly. Thus, ClpA recognizes an N-terminal signal in RepA beginning in the vicinity of amino acids 10–15. Moreover, peptides corresponding to RepA amino acids 4–13 and 1–15 inhibit interactions between ClpA and RepA. We constructed fusions of RepA and green fluorescent protein, a protein not recognized by ClpA, and found that the N-terminal 15 amino acids of RepA are sufficient to target the fusion protein for degradation by ClpAP. However, fusion proteins containing 46 or 70 N-terminal amino acids of RepA are degraded more efficiently in vitro and are noticeably stabilized in vivo in clpAD and clpPA strains compared with wild type.

Clp/Hsp100 ATPases are a ubiquitous family of proteins that participate in protein unfolding and remodeling associated with many cellular functions including DNA replication, tolerance to heat stress, control of gene expression, and protein degradation (1–3). For example, Escherichia coli ClpA dissociates inactive dimers of plasmid P1 RepA into monomers, thereby activating specific DNA binding by monomeric RepA (4, 5). Similarly, ClpX of E. coli disassembles protein complexes of MuA transposase bound to DNA (6, 7) and aggregates of λ O protein (8). It also dissociates inactive dimers of the initiator protein of plasmid RK2 into active monomers (9). Both in vivo and in vitro Hsp104, a yeast Clp ATPase, participates in disaggregating heat-denatured proteins in combination with the yeast DnaK chaperone system (10, 11). Likewise, the E. coli Hsp104 homolog, ClpB, dissociates aggregates in combination with DnaK, DnaJ, and GrpE (12–14). Some of the Clp ATPases play a role in degradation when associated with a proteolytic component that alone is unable to degrade large polypeptides (15, 16). ClpA or ClpX can associate with ClpP forming ClpAP and ClpXP, which degrade specific proteins. For example, ClpXP and to a lesser extent ClpAP degrade SsrA-tagged protein fragments (17–19). When protein synthesis stalls, because the messenger RNA is damaged and lacks a stop signal, a small RNA, SsrA, is directed to the ribosome and codes for the addition of 11 amino acids to the end of the protein fragment. This 11-amino acid peptide tags the protein for degradation by ClpXP and ClpAP.

Structural studies have shown that Clp ATPases self-assemble into oligomeric rings in the presence of ATP or nonhydrolyzable ATP analogs (20–24). When associated with a proteolytic component, the ATPase rings are at either or both ends of the proteolytic core forming a structure resembling the eukaryotic 26 S proteasome (20). The crystal structures of two proteolytic components, ClpP and HsIV of E. coli, show that the proteolytic sites are in an internal chamber of stacked rings of identical subunits resembling the proteolytic core of the 26 S proteasome (25–28). Access to the proteolytic chamber appears to be through narrow pores at either end of the stacked rings. However, the pores are not large enough to allow the passage of a native globular protein. The crystal structure of an E. coli Clp ATPase, HsIu, shows a ring structure with 6-fold symmetry made up of individual subunits that are strikingly similar to their counterparts in a classic AAA-ATPase, N-ethylmaleimide-sensitive fusion (29–31). From the biochemical and structural data, it was proposed that the ATPase components flanking the proteolytic core specifically bind and unfold the substrate and then translocate the unfolded polypeptide through the small pore into the proteolytic chamber (32). Recent studies support this model. Both ClpA and ClpX unfold proteins (33–36), and both translocate substrates from binding sites on the ATPase component to ClpP (34, 36, 37).

The substrate specificity of the protease is determined by the ATPase component. For several substrates, including MuA (38), Mu vir repressor (39), and SsrA-tagged polypeptides (17), sites in the C-terminal sequences of the substrate are primarily responsible for recognition by Clp ATPases. For others, including certain β-galactosidase fusion proteins bearing hydrophobic N-terminal amino acids (40, 41), HemA (42), UmuD’ (43), and λ O (44), recognition is through sites in the N-terminal region. It is still unclear what the requirements are for substrate recognition, and efforts to align sequences of known specific substrates have not led to the identification of a simple linear recognition motif. To gain a better understanding of substrate recognition, we characterized a site in RepA that is responsible for the interaction of RepA with ClpA. We found that ClpA recognizes an N-terminal site in RepA that begins in the vicinity of amino acids 10–15. We also found that the first 15 amino acids of RepA, when fused to an otherwise stable protein, are necessary and sufficient to target the fusion protein for degradation by ClpAP.

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EXPERIMENTAL PROCEDURES

Materials—ATP and ATP[S] were obtained from Roche Molecular Biochemicals. Restriction endonucleases and DNA modifying enzymes were obtained from New England BioLabs. Polymerase chain reaction reagents were obtained from PerkinElmer Life Sciences. Peptides were synthesized, purified, and analyzed by mass spectrometry by Research Genetics, Inc.

Plasmids and Strains—Plasmids expressing the N-terminal regions of RepA were constructed by BstBI or MluI cleavage of plasmid pRE-RepA, a plasmid carrying the P1 repA gene cloned under the control of the A ρE promoter (45). Following cleavage, the ends were filled in and ligated in the presence of a DNA linker containing a termination codon. Plasmids expressing N-terminal deletions of RepA were constructed by generating appropriate repA polymerase chain reaction fragments containing 5′ and 3′ EcoRI and BamHI sites, respectively, and then ligating the fragments into pET11a (Stratagene). RepA sequences were verified by DNA sequencing.

Plasmids expressing RepA(1–15)GFP, RepA(1–46)GFP, RepA(1–70)GFP, and RepA(1–70)GFPuv under the control of the arabinose-inducible pBAD promoter were constructed as described (35). The sequences of the gene fusions were verified by DNA sequencing. E. coli clpAΔ, clpXΔ, and clpPΔ derivatives of a MC4100 ara strain were kindly provided by Susan Gottesman (National Institutes of Health) (35).

Proteins and DNA—P1 RepA (46), ClpA (47), ClpP (47), and RepA (1–70)GFP (35) were purified as described. RepA(1–15)GFP was purified using the method developed for the isolation of RepA(1–70)GFP (35). RepA deletion proteins were isolated by the method described for RepA (46). GroEL trap [GroEL(D87K)] was prepared as described (48). (35). RepA deletion proteins were isolated by the method described for the isolation of RepA(1–70)GFP monomers. 3H-Labeled RepA(1–70)GFP monomers, H-Labeled oriP1 plasmid DNA (3800 cpm/ fmol) was prepared as described (49).

RepA Activation Assay—Reaction mixtures contained (in 20 μl) buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol (v/v)), 1 mM ATP, 10 mM MgCl2, 5 μM RepA, 100 μg/ml bovine serum albumin, 0.5 pmol ClpA, and 0.1 pmol RepA, unless indicated otherwise. After 10 min at 23 °C, unless indicated otherwise, the mixtures were chilled to 0 °C. Calf thymus DNA (1 μg) and 11 fmol of [3H]oriP1 plasmid DNA were added. After 5 min at 0 °C, the mixtures were filtered through nitrocellulose filters, and the retained radioactivity was measured.

RepA-ClpA Complex Formation—Reaction mixtures (80 μl) contained buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol (v/v)), 1 mM ATP, 10 mM MgCl2, 5 μM RepA, 100 μg/ml bovine serum albumin, 0.5 pmol ClpA, and 0.1 pmol RepA, unless indicated otherwise. After 20 min at 23 °C, unless indicated otherwise, the mixtures were chilled to 0 °C. Triton X-100 (0.01% w/v), and bovine serum albumin (100 μg/ml) were added to the mixtures (final volume, 100 μl), and complex formation was quantitated by measuring radioactive retention on microcon 100 filters (Amicon) as described previously (5).

RepA Degradation Assays—To detect proteolysis as measured by acid solubilization of labeled RepA, reaction mixtures were assembled in 20 μl of buffer A containing 100 μg/ml bovine serum albumin, 10 μM MgCl2, 5 mM ATP, 0.8 pmol ClpA, 0.8 pmol ClpP, and 0.9 pmol [3H]RepA, unless indicated otherwise. The mixtures were incubated at 24 °C for 15 min or the times indicated. Trichloroacetic acid was added to 20% (w/v), and degradation was quantitated by measuring acid soluble radioactivity. In the [3H]RepA preparations used, <1% of the label was trichloroacetic acid soluble in the absence of ClpAP.

To measure proteolysis by the decrease in fluorescence of GFP or RepA-GFP fusion proteins, reaction mixtures were assembled in 100 μl of buffer A, 20 mM MgCl2, 10 mM ATP, 20 mM creatine phosphate, 6 μg of creatine kinase, 160 pmol of ClpA, 200 pmol of ClpP, and 40 pmol of GFP or a RepA-GFP derivative. The mixtures were incubated at 25 °C for the times indicated. The decrease in relative fluorescence was measured as a function of time with excitation at 395 nm and emission at 515 nm using a PerkinElmer Life Sciences LS50B luminescence spectrophotometer equipped with a well plate reader.

To quantify degradation by SDS-PAGE, 20% trichloroacetic acid (w/v) was added to reaction mixtures after a 20-min incubation as described above. The trichloroacetic acid pellets were analyzed by SDS-PAGE, and the amount of substrate was quantitated by densitometry.

 Results

The N-terminal Portion of RepA Is Necessary for Recognition by ClpA—We wanted to investigate the mechanism of substrate recognition by ClpA by characterizing the interaction of RepA with a known substrate. RepA was chosen because it is both a substrate for remodeling by ClpAP and for degradation by ClpAP. Importantly, RepA has a high affinity for ClpA with an apparent Kd of about 2 nM. To roughly define the region of RepA that contains the ClpA recognition signal, we cloned and expressed the N- and C-terminal portions of RepA separately. Degradation of the purified truncated proteins was then measured in reaction mixtures with ClpAP in vitro. We observed that a derivative lacking the N-terminal 49 amino acids of RepA, RepA(50–286), was poorly degraded by ClpAP (Fig. 1). In contrast, when the C-terminal 106 amino acids were removed, ClpAP degraded the truncated protein, RepA(1–180), at a rate similar to full-length RepA (Fig. 1). These results suggest that a signal necessary for ClpA recognition is located within the first 49 amino acids of RepA.

To more precisely identify the recognition signal in RepA, we constructed, expressed, and purified RepA proteins deleted for the N-terminal portion of RepA in increments of 5 amino acids (Fig. 2). The RepA derivatives were then tested as potential substrates for degradation by ClpAP (Fig. 3). ClpAP degraded RepA derivatives lacking the first 5 or first 10 amino acids, RepAΔ5 and RepAΔ10, at similar rates as wild type RepA. However, proteins deleted for the N-terminal 15 or 20 amino acids, RepAΔ15 and RepAΔ20, were degraded at 4- and 8-fold slower rates, respectively, than wild type RepA, suggesting that a ClpA recognition site exists near the N terminus of RepA. One of the deletion proteins, RepAΔ5, was degraded at a rate similar to wild type, indicating that another ClpA recognition site in RepA is exposed by the deletion (data not shown).

We also tested whether ClpA could activate the DNA binding activity of the various RepA deletion derivatives as it does wild type RepA by converting inactive RepA dimers into active RepA monomers (Fig. 4). ClpA activated both RepAΔ5 and RepAΔ10 as it did wild type RepA. However, RepAΔ15 was poorly activated by ClpA. To eliminate the possibility that Δ15 was recognized by ClpA but bound DNA poorly, RepA derivatives were

FIG. 1. Degradation of N- and C-terminal truncated RepA proteins by ClpAP. ClpA (20 pmol) and ClpP (9 pmol) were incubated with 90 pmol of RepA wild type (circles), RepA(1–180) (squares), or RepA(50–286) (triangles) at 24 °C in 240-μl reaction mixtures containing buffer A, 6 mM MgCl2, and 2 mM ATP. At 0, 5, 10, 20, and 40 min, 5 μg GroEL trap. Degradation was quantitated by densitometric analysis of Coomassie-stained gels.

RepA-GFP Unfolding Assays—Protein unfolding was measured as described above for the fluorescent proteolysis assay, but ClpP was omitted. Reactions were carried out in the absence and presence of 2.5 μM GroEL trap.

The abbreviations used are: ATP[S], adenosine-5′-O-3-thiotriphosphate; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

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treated with guanidine HCl using conditions known to activate DNA binding by chemically converting RepA dimers to monomers (50). We found that guanidine-treated RepA bound DNA with the same high affinity as similarly treated wild type, RepAΔ5, and RepAΔ10 (Fig. 5). Taken together the results from the studies of RepA deletion derivatives suggest that a site necessary for ClpA recognition resides in the N-terminal portion of RepA beginning within amino acids 10–15.

**Peptides Corresponding to the N-terminal Portion of RepA Are Specifically Recognized by ClpA**—To determine the limits of the ClpA recognition site in RepA, we compared the ability of various RepA peptides to compete with wild type RepA for activation by ClpA and degradation by ClpAP. We observed that a peptide corresponding to amino acids 1–15 of RepA (MNQSFISDILYADIE) significantly inhibited both RepA activation by ClpA (Fig. 6) and degradation by ClpAP (Fig. 7A). A smaller N-terminal peptide corresponding to amino acids 4–13 was also inhibitory (Fig. 7A). In contrast peptides corresponding to amino acids 1–10 and 11–20 did not inhibit activation or degradation significantly. A peptide corresponding to the C-terminal 15 amino acids of RepA, RepA(272–286), had no significant effect on either reaction. Likewise, an internal RepA peptide, RepA(36–50), did not inhibit activation or degradation, although RepAΔ35 was degraded by ClpAP. Experiments measuring inhibition of degradation in a single cycle of substrate binding showed again that RepA1–15 inhibited degradation and that the other peptides tested did not (Fig. 7B).

We tested the RepA peptides as competitive inhibitors of complex formation between ClpA and RepA to eliminate the possibility that the peptides were inhibiting degradation by ClpP rather than recognition by ClpA. Complexes of [3H]RepA and ClpA were assembled in the presence of ATPγS and various concentrations of the peptides. After a short incubation, free [3H]RepA was separated from ClpA-RepA complexes by ultrafiltration, and the amount of [3H]RepA associated with ClpA was quantitated. We found that the RepA(1–15) peptide inhibited ClpA-RepA complex formation (Fig. 8). RepA(1–10), RepA(36–50), and RepA(272–286) had very little effect.

In control experiments, RepA peptides were tested for their ability to inhibit degradation of another ClpAP substrate, α-casein. RepA(1–15) inhibited α-casein degradation, whereas RepA(36–50) and RepA(277–286) had no effect (data not shown). In another control experiment, we measured the effect of the peptides on degradation by ClpXP. Because RepA is neither activated by ClpX nor degraded by ClpXP (4), we expected and observed that the RepA(1–15) peptide had no effect on degradation of α casein by ClpXP (data not shown). The peptide inhibition experiments demonstrate that ClpA specifically binds a site within the first 15 amino acids of RepA and more precisely within amino acids 4–13.

**The N-terminal 15-Amino Acid Peptide of RepA Is Sufficient to Target GFP for Recognition by ClpA**—We wanted to know whether the N-terminal portion of RepA could tag an otherwise unrecognized protein for recognition by ClpA. GFP was chosen...
for these experiments, because it is not recognized by ClpA (33, 35). Several gene fusions between repA and GFP were constructed and expressed under control of the ara BAD promoter. The GFP gene cloned in the same vector was used as a control. The resulting plasmids produced fusion proteins containing the N-terminal 15, 46, or 70 amino acids of RepA fused to the N-terminal end of full-length GFP, referred to as RepA(1–15)GFP, RepA(1–46)GFP, and RepA(1–70)GFP, respectively. The plasmids were introduced into araΔ derivatives of E. coli wild type, clpAΔ, clpPΔ, or clpXΔ strains and grown in the presence of arabinose. It could be seen by visible light that the wild type, clpAΔ, and clpPΔ strains grew better than the clpXΔ strain, independent of the plasmid used (Fig. 9, A, C, E, and G). When viewed under UV light, colonies of all four strains expressing GFP were fluorescent (Fig. 9B). In contrast, colonies of clpAΔ and clpPΔ strains expressing RepA(1–70)GFP were significantly more fluorescent than the wild type and clpXΔ, suggesting that the fusion protein was stabilized by the absence of ClpAP but not ClpXP (Fig. 9D). Similarly, clpAΔ and clpPΔ strains expressing RepA(1–46) were more fluorescent than the clpXΔ strain and slightly more fluorescent than wild type (Fig. 9F). The clpAΔ strain expressing RepA(1–15)GFP was more fluorescent than the clpXΔ and wild type strains; however, the clpPΔ strain was indistinguishable from the wild type strain (Fig. 9H). These results suggest that in vivo the N-terminal portion of RepA targets GFP for degradation by ClpAP and not by ClpXP and that 46 or more N-terminal amino acids of RepA are required.

We purified RepA(1–15)GFP and RepA(1–70)GFP and measured the disappearance of fluorescence in the presence of ClpAP in vitro as an indication of degradation (Fig. 10). The fluorescence of both fusion proteins decreased following incubation with ClpAP and ATP. With these conditions, the fluorescence of GFP was unchanged (data not shown and Refs. 33 and 35). However, the fluorescence of RepA(1–15)GFP decreased more slowly than that of RepA(1–70)GFP, suggesting that it had lower affinity for ClpA than RepA(1–70)GFP. Degradation was confirmed by quantitating the disappearance of the proteins by SDS-PAGE. After a 20-min incubation with ClpAP, using conditions shown in Fig. 10, 65% of the RepA(1–70)GFP and 47% of the RepA(1–15)GFP were degraded. Thus in vitro degradation of RepA(1–70) confirms the in vivo results shown above. These results further show that the first 15 amino acids of RepA are both necessary and sufficient to tag GFP for recognition by ClpA and degradation by ClpAP.

The difficulty in detecting in vivo degradation of RepA(1–15)GFP is best explained by ClpA having a lower affinity for RepA(1–15)GFP compared with RepA(1–70)GFP. To compare the affinity of RepA(1–15)GFP and RepA(1–70)GFP for ClpA by another assay, we measured steady state unfolding by ClpA. The Horwich laboratory showed that ClpA catalyzes unfolding of a GFP derivative that contains the 11-amino acid SsrA tag fused to the C-terminal end of GFP, GFP-SsrA (33). Unfolding...
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was measured as a decrease in fluorescence dependent on ClpA and ATP. GroEL trap (48), a mutant form of GroEL that binds unfolded proteins but does not release them, was required in the reaction mixtures to sequester unfolded GFP-SsrA released by ClpA, thereby preventing the spontaneous refolding of GFP-SsrA (33). Global unfolding was confirmed by deuterium-hydrogen exchange experiments (33). RepA(1–15)GFP, like GFP-SsrA, showed a less than 5% decrease in fluorescence upon incubation with ClpA and ATP (Fig. 11A). With the addition of GroEL trap, a 30% decrease in fluorescence was seen because of sequestration of unfolded RepA(1–15)GFP (Fig. 11B). In contrast, when RepA(1–70)GFP was incubated with ClpA and ATP, the fluorescence decreased about 40% (Fig. 11A). RepA(1–70)GFP was not irreversibly “trapped” by ClpA and regained its original fluorescence after about 30 min, indicating that after ATP was depleted, the steady state unfolding reaction ceased (35). When GroEL trap was included in the reaction mixtures with RepA(1–70)GFP and ClpA, fluorescence decreased 60% (Fig. 11B). As expected, fluorescence was not regained with time, because GroEL trap irreversibly bound the unfolded protein. Thus RepA(1–70)GFP has a higher affinity for ClpA than RepA(1–15)GFP, such that in the steady state much of the RepA(1–70)GFP is involved in cycles of binding, unfolding, releasing, and refolding. These results suggest that other signals present in RepA(1–70)GFP significantly increase the affinity of the fusion protein for ClpA.

**DISCUSSION**

We have shown that a signal in the N-terminal 15 amino acids of RepA tags RepA for recognition by ClpA. Interestingly,
the site is near but not at the N terminus, beginning in the vicinity of amino acids 10–15. This result suggests a slightly different type of recognition site than that reported by Tobias et al. (40), who found that the addition of certain single amino acids, including leucine and phenylalanine, to the N terminus of β-galactosidase tags the protein for degradation by ClpAP. In vivo, leucine, phenylalanine transfer RNA-protein transferase specifically adds leucine or phenylalanine to proteins bearing an N-terminal arginine or lysine, thus targeting them for degradation by ClpAP (41). For HemA, another ClpAP substrate, the recognition signal resides within the N-terminal 18 amino acids, but a more precise location has not been determined (42). For SsrA-tagged proteins, both ClpAP and ClpXP recognize the C-terminal 11-amino acid addition (18, 36). It has been shown that changing the last two C-terminal residues of SsrA from Ala-Ala to Asp-Asp destroys recognition by both ClpA and ClpXP, implicating the two terminal amino acids in recognition (43). Although the mechanism of ATP-dependent degradation is becoming clearer, important questions remain unanswered, and the working model remains speculative.

Acknowledgments—We thank Susan Gottesman for constructing strains for us, Arthur Horwich for giving us the GroEL trap expression plasmid, and Yawan Bai, Susan Gottesman, and Michael Maurizi for helpful discussions.

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