Recognition, Targeting, and Hydrolysis of the λ O Replication Protein by the ClpP/ClpX Protease*

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The Hsp100 family of protein ATPases was originally identified in *Escherichia coli* through the roles of individual ATPases as regulatory components of certain ATP-dependent serine proteases, such as ClpP (for reviews see Refs. 1–3). For example, the ClpA and ClpX family members were isolated as factors that enabled the ClpP catalytic component to hydrolyze certain polypeptide substrates in an ATP-dependent manner (4–7). On its own, the ClpP protease degrades only very small peptides, and such hydrolysis does not depend on ATP (8, 9). In (4–7). On its own, the ClpP protease degrades only very small peptides, and such hydrolysis does not depend on ATP (8, 9). In

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1 The abbreviations used are: ATP:S, adenosine 5’-O-(3-thiotriphos- phate); ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

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It has previously been established that sequences at the C termini of polypeptide substrates are critical for efficient hydrolysis by the ClpP/ClpX ATP-dependent protease. We report for the bacteriophage λ O replication protein, however, that N-terminal sequences play the most critical role in facilitating proteolysis by ClpP/ClpX. The N-terminal portion of λ O is degraded at a rate comparable with that of wild type O protein, whereas the C-terminal domain of O is hydrolyzed at least 10-fold more slowly. Consistent with these results, deletion of the first 18 amino acids of λ O blocks degradation of the N-terminal domain, whereas proteolysis of the O C-terminal domain is only slightly diminished as a result of deletion of the C-terminal 15 amino acids. We demonstrate that ClpP retains its capacity to bind to the N-terminal domain upon deletion, whereas exposure on the substrate surface may be induced by the binding of ClpX.

Clp/ClpA-mediated hydrolysis of peptides of moderate length is stimulated. This suggests that interactions between ClpA and ClpP, promoted by ATP hydrolysis, are essential for ClpP ATPase activity. In the presence of ATP, the Clp/ClpA complex is activated, and ATP hydrolysis per se is required to achieve progressive degradation of larger polypeptides.

Hsp104, a *Saccharomyces cerevisiae* homologue of *E. coli* ClpB, has been shown to be essential for cellular thermostolerance (10). Interestingly, overexpression of the molecular chaperone Hsp70 largely reverses the temperature sensitivity exhibited by the hsp104 mutant (11). These and other findings support the idea that members of the Clp family of ATPases can perform certain chaperone functions in the absence of their partner protease subunit (12). This hypothesis has been confirmed by recent experimental findings, namely: (i) that Hsp104 participates in the disaggregation of partially damaged or aggregated protein structures (13, 14) as well as modulates the conformational transition between normal and altered forms of prion-like factors (15), (ii) that bacterial ClpA protein substitutes in *vitro* for the DnaK/DnaJ/GrpE chaperone machine in activation of the Agg P1 RepA replication protein for binding to its recognition sites in the P1 replication origin (16, 17), and (iii) that ClpX protein enhances the binding of the λ O replication initiator to the λ replication origin (18) and promotes the binding of the RK2 plasmid-encoded TrpA protein to the oriRK2 sequence (19). Yet perhaps the most convincing evidence for the notion that Clp ATPases have intrinsic chaperone activity comes from investigations of the bacteriophage Mu DNA replication system. Initially it was discovered that deletion of the *E. coli* clpP gene (but not the clpP gene) resulted in a total inactivation of Mu DNA replication in vivo (20). Later, studies of the mechanism of Mu DNA replication in a reconstituted multiprotein system showed that ClpX acts at a step prior to the inhibition of DNA replication to promote the disassembly of an extremely stable MuA transposase nucleoprotein complex (21–23). It is suggested that ClpX-mediated release of MuA transposase from a transposition intermediate triggers initiation of Mu DNA replication by providing access of host replication proteins to Mu DNA ends (24).

Both the protease and chaperone activities of the Clp ATPases depend on ATP hydrolysis. In the presence of ATP, the ClpA or Hsp104 proteins individually oligomerize to give rise to a hexameric, ring-like structure (25–27). Electron microscopic studies have shown that ClpA or ClpX rings bind to the 7-fold symmetric ClpP component, which itself is arranged in a barrel-like double-ring structure. These interactions produce an overall structure that highly resembles the eukaryotic 26 S proteasome (28–30).

It has been suggested that ClpA recognizes relatively un-
folded proteins, whereas ClpX is apparently much more specific. For example, ClpX is known to recognize the λ O (6, 7), P1 Phd (31), Mu Reppressor (32), MuA transposase (21), σ’ (33), and UmuD’ (34) proteins, each of which seemingly exists in a native, properly folded state. These findings support the hypothesis that the Clp ATPases are specificity factors that are capable of recruiting different protein substrates and, subsequently, transferring them to the ClpP subunit for proteolysis (6, 7). It is still not certain which sequences or protein structures in substrate proteins are recognized by the Clp/Hsp100 family (2, 35). For several protein substrates, e.g. MuA transposase (36), mutants of bacteriophage Mu repressor (32), or SsrA-tagged polypeptides (37), it has been shown that C-terminal sequences are primarily responsible for their targeting to the ClpP/ClpX protease. Recently, the presence of PDZ-like domains has been detected in ClpX and other Clp/Hsp100 family members. These structural motifs have been suggested to mediate the binding specificity of these proteins to different protein substrates (36). Furthermore, it was postulated that the initiating event during substrate selection by ClpX is the formation of a complex between ClpX and exposed C-terminal residues of a substrate protein (36). We decided to test the universality of this proposed mechanism using wild type and deletion mutant forms of the λ O replication initiator protein. Wild type O protein, a highly effective substrate of the ClpP/ClpX protease, is composed of N- and C-terminal structural domains, which are connected by a relatively unstructured linker region (38). Contrary to published results with MuA transposase, mutants of Mu repressor, and SsrA-tagged polypeptides, we demonstrated the importance of C-terminal sequences for hydrolysis by the ClpP/ClpX protease, we provide evidence that it is the N-terminal portion of the λ O replication protein that is primarily required for its efficient hydrolysis by this ATP-dependent protease. In addition, we also demonstrate that stable binding of substrate polypeptides to ClpX is not sufficient to ensure proteolysis by ClpP/ClpX. We suggest that in addition to a ClpX-binding site, special N-terminal or C-terminal sequences must be present in the ClpP/ClpX substrate polypeptide.

MATERIALS AND METHODS

Plasmids and Mutagenesis—Construction of plasmids that overexpress wild type λ O protein (plasmid pRLM77) or certain truncated versions of O (O1–126/181–299) from plasmid pRLM166; O1–162 from plasmid pRLM84; O1–139 from plasmid pRLM146; O19–110 from plasmid pRLM147; O1–110 from plasmid pRLM150; and O19–110 from plasmid pRLM151) have been described (38). Plasmids that overexpress O150–299 (plasmid pRLM216) and O19–299 (plasmid pRLM312) will be described in detail elsewhere. In brief, plasmid pRLM77 contains a 1.5-kilobase pair Alul fragment harboring the λ O gene inserted into the multiple cloning site present on expression plasmid pRLM76 (39). Plasmid pRLM84 was constructed from plasmid pRLM77 by digesting the plasmid at the unique EcoRI site, located within the O gene, filling in the cohesive end with the Klenow fragment of DNA polymerase I, and religating. This creates a 4-base pair duplication that results in an in-frame chain termination codon. Plasmid pRLM166 was constructed by digesting plasmid pRLM77 with EcoRI, treating the linearized DNA with Bal31 nuclease, and religating. Plasmids that overproduce truncated λ O proteins were identified and sequenced so that the end points of each internal deletion in the O gene could be defined. A plasmid producing an O protein, deleted for amino acid residues 127–180, was named pRLM186. Plasmids pRLM146, pRLM147, pRLM150, pRLM151, and pRLM216, and pRLM311 each contain a segment of the O gene generated by PCR-mediated amplification of DNA sequences carried in plasmid pRLM4 (40). The primers used at one end for PCR amplification carried in order (5’ to 3’) were Bcll HI restriction site, a strong ribosome-binding site from gene 10 of phage T7, a properly spaced ATG initiation codon, followed by the desired O coding sequence. The primers used for synthesis of the complementary strand contained in order (5’ to 3’) were XbaI, PstI, and SalI restriction sites, two tandem chain termination anti-codons, and a sequence complementary to the codons for the desired new C terminus. The PCR-amplified DNA preparations were digested with both BamHI and PstI or with both BamHI and Sall. These DNA fragments were subsequently inserted by ligation between BamHI and PstI polylinker sites present on plasmid pRLM76 (pRLM146, pRLM147, pRLM150, and pRLM151) or between the BamHI and Sall polylinker sites present on plasmid pRLM156 (41) (pRLM216 and pRLM311). The expression vector for O(19–299), pRLM312, was constructed by ligating the appropriate BamHI/Sall fragment of pRLM311 between the BamHI and Sall sites of pE2T(+)(Novagen). All plasmid sequences derived from PCR amplification were verified by DNA sequence analysis using the dyeexodeoxyribonucleotide triphosphate chain termination method.

For mutagenesis of a region encompassing the putative ClpX consensus recognition site in the C-terminal domain of λ O, the 488 base pairs BamHI-PstI fragment of pRLM216 was cloned into BamHI-PstI pGEM3Zf(-) vector (Promega) to give plasmid pMZ2. This plasmid was subsequently transformed into the bacterial strain CJ236 due ung, and single-stranded, uracil-containing DNA was produced by mobilization with helper phage VCSM13 (Stratagene), according to the method of Kunkel et al. (42). Primer extension reactions were performed in the presence of 5% Me2SO in the reaction mixture. The mutations resulting in λ O150–299–D287A and the C-terminal truncation λ O150–284, missing the final 15 amino acids of O, were constructed by oligonucleotide-directed mutagenesis using the primers: D287A 5′-CTGGTTTTGCTCACGGGGCGATTGTGTGTCG-3′ and PstI-stop284 5′-CTGTTGTGTGCTCACGGGGCGATTGTGTGTCG-3′, respectively. Candidate mutant plasmids were initially screened for loss of a TaqI restriction site (O150–299–D287A) or for a specific BamHI-PstI digestion pattern (O150–284). The BamHI-PstI inserts of positive clones were verified by the dyeexodeoxynucleotide sequencing technique using Sequenase version 2.0. Finally, the BamHI-PstI inserts were excised and recloned into pRLM4 (43) to yield the expression plasmids pMZ5 and pMZ6, encoding λ O150–299–D287A and λ O150–284, respectively.

Proteins—Highly purified proteins (95% or greater purity) were used in all experiments described in this paper. The ClpX-overproducing strain was the kind gift of Drs. Satish Raina and Dominique Missiakas (University of Geneva). The ClpX and ClpP proteins were purified as described by Wójcikiewicz et al. (6).

The wild type λ O protein, O(1–299), deletion mutant proteins harboring the N-terminal domain of O (λ O1–162, λ O1–139, and λ O19–139), the central deletion mutant protein, λ O126–181–299, and λ O19–299 were each purified as described by Roberts and McMacken (44). Polypeptides containing the C-terminal domain of O, namely λ O150–284, and λ O150–299–D287A were purified as follows. After cell lysis (24 g of cells) in the presence of lysozyme and spermidine and centrifugation (44), the supernatant (120 ml) was mixed with ammonium sulfate (0.3 g/ml supernatant) and stirred for 30 min at 4 °C. Following centrifugation at 70,000 g for 30 min at 4 °C, the pellet was resuspended and dialyzed against buffer A (50 mM Tris/HCl, pH 7.4, 0.1 M KCl, 0.1 mM EDTA, 10% [v/v] glycerol, 5 mM dithiothreitol, 0.02% Triton X-100) and applied to a Pharmacia Q-Sepharose (2.5 × 14 cm) column that had been equilibrated with buffer A. The void volume fractions were pooled (60 ml) and applied to a Whatman P11 phosphocellulose column (1.5 × 8 cm) that was equilibrated with buffer A. Bound proteins were eluted with a linear gradient (120 ml) of 0.1–0.7 M KCl in buffer A. Fractions containing significant quantities of O protein were pooled (35 ml) and applied to a hydroxylapatite column (1.5 × 5 cm) that was equilibrated with buffer A. Bound protein was eluted with a linear gradient (120 ml) of 0.1–0.7 M KCl in buffer A. The fractions containing pure protein were pooled, frozen, and stored at −80 °C prior to use.

The λ O1–110 N-terminal fragment of λ O was purified from the membrane fraction of the crude lysate. After cell lysis (40 g of cells) with lysozyme and centrifugation of the lysate at 70,000 × g for 60 min at 4 °C, the membrane-containing pellet was resuspended in 50 ml of buffer B (50 mM Tris/HCl, pH 7.4, 0.2 M KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% [v/v] glycerol, 0.04% Triton X-100, 4 mM urea) and sonicated. Following gently overnight shaking, the suspension was centrifuged at 70,000 × g for 30 min at 4 °C. The supernatant (70 ml) was then applied to 2 liters of buffer C (50 mM Tris/HCl, pH 7.4, 0.2 M KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% [v/v] glycerol, 0.04% Triton X-100, 4 mM urea) and applied to a P11 phosphocellulose column (2.5 × 14 cm) equilibrated with buffer C. The column was washed with several column volumes of buffer C, and the bound proteins were subsequently eluted with a linear gradient (400 ml of KCl. 0.2–0.8 M in buffer C). Fractions containing significant amounts of the N-terminal domain of λ O protein were pooled, dialyzed against buffer C, and applied to a 6-ml Resource MonoS
column (Pharmacia) that was equilibrated with buffer C. The bound protein was eluted with a 50-ml KCl gradient (0.2–0.5 M in buffer C) and was stored at −80 °C.

O(19–110) protein was purified as described above for O(1–110), except that a hydroxylapatite column (1.2 × 4.8 cm) was substituted for the Resource MonoS column during the final purification step. A linear gradient (50 ml) from 0.1 to 0.7 M KCl in buffer C was used to elute bound protein from the column. Fractions containing homogeneous O(19–110) were frozen in liquid nitrogen and stored at −80 °C. The N-terminal sequences of O and all purified O deletion mutant proteins were determined using an ABI 473A Applied Biosystems Microsequencer system.

Protease Assays—The standard protease assay (150 μl) was carried out in 20 mM HEPES, pH 7.6, 10 mM MgAc2, 0.5% Brij, and 10 mM ATP. Each assay included 5 μg of ClpP, 5 μg of ClpX, and 5 μg of the appropriate substrate polypeptide. The reaction mixture was incubated at 30 °C. At desired times, 25-μl portions of the reaction mixture were withdrawn and processed by 12.5% SDS-polyacrylamide gel electrophoresis.

Protein-Protein Interaction Assays—The sensitive ELISA assay used for monitoring protein-protein interactions has been previously described in detail by Wawrzynow et al. (18). The buffer B used in these experiments contained 25 mM HEPES/KOH, pH 7.6, 150 mM KCl, 25 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 2.5% (v/v) glycerol, 0.05% Triton X-100, and 1 mM ATP.

Single-stranded DNA Replication Assay—λ single-stranded DNA replication assays were performed as described (45, 46) with modifications as follows. M13mp9 viral DNA (165 ng) was used as the single-stranded DNA template, and reaction mixtures also contained 90 ng of E. coli GrpE protein. DNA synthesis was measured after a 20-min incubation at 30 °C.

RESULTS

The N-terminal Portion of λ O Is Important for the ClpP/ClpX-dependent Hydrolysis of λ O—It has previously been shown that the ClpX and ClpA ATPases recognize specific protein substrates, leading to selective ClpP-dependent proteolysis (6, 18). Subsequent studies led to the hypothesis that sequences at the C termini of substrates of the ClpP/ClpX and ClpP/ClpA proteases are required for targeting the substrate polypeptide to the ClpX or ClpA ATPases (36, 37). We decided to investigate whether this C-terminal substrate recognition mechanism applies uniformly to all physiological substrates of ClpX. We tested wild type λ O replication protein, which is known to be a particularly good substrate of the ClpP/ClpX protease both in vivo and in vitro, and various truncated derivatives of O (Fig. 1) for their sensitivity to hydrolysis by the ClpP/ClpX protease. We were surprised to find that the C-terminal domain of λ O protein, represented by λ O(150–299), is hydrolyzed much more slowly by the ClpP/ClpX protease than either wild type λ O(1–299) or a N-terminal domain fragment, λ O(1–162) (Figs. 2 and 3). Control experiments indicate that both the N- and C-terminal domains of λ O are apparently folded correctly, even in the absence of the other domain, because each individual domain retains its relevant biological activity. Thus, the λ O(1–162) polypeptide, which contains the origin recognition elements of O, has been shown to bind specifically to the O binding sites present in oriC DNA (38). Likewise, the C-terminal O(150–299) fragment remains capable of functionally interacting with the λ P-DnaB complex to support the transfer of DnaB helicase onto DNA (45), as indicated by the significant levels of DNA replication obtained (Table I) when the O(150–299) protein replaces wild type λ O protein in the λ single-stranded replication reaction (45, 46). The N-terminal origin recognition domain alone is incapable of supporting this single-stranded DNA replication reaction (Table I).

To exclude the possibility that the central portion of the λ O protein provides the structural motif that permits binding to ClpX, we tested a λ O mutant with a large central deletion, λ O(1–126/181–299). This O deletion mutant protein retains significant functional activity in an in vitro λ DNA replication system (38). λ O(1–126/181–299) is almost as sensitive to ClpP/ClpX-mediated proteolysis as wild type λ O (Fig. 3). In addition, the deletion of the C-terminal 15 amino acids from the λ O(150–299) polypeptide, resulting in an O(150–284) truncated fragment, only slightly alters its sensitivity to proteolysis (Fig. 3). This result indicates that the λ O protein behaves differently than the MuA transposase or a related fusion protein, Arc-MuA, where deletion of the last 8 amino acids from their C termini almost completely stabilized MuA against ClpP/ClpX-
dependent proteolysis (36). It was noted previously that the aspartic acid at position 287 of O is a part of the LDL/A consensus sequence present in all known ClpX protein substrates (2). To test this putative recognition sequence, we introduced a point mutation, D287A, in the O(1–110) polypeptide. However, contrary to expectation, this sequence alteration did not substantially affect the rate of degradation of the O C-terminal domain (Fig. 3).

The smallest O protein fragment tested here that was efficiently hydrolyzed by the ClpP/ClpX protease is the λ O(1–110) N-terminal polypeptide (Fig. 3). We discovered, however, that further deletion of the first 18 amino acids (MTNTAKILNFGRGNFAGQ) from this truncated substrate, resulting in the O(1–110), O(1–126), O(1–299) polypeptide. Since the consensus sequence present in all known ClpX protein substrates (2)

**Table I**

| O protein derivative | DNA synthesis (pmol) |
|----------------------|----------------------|
| None                 | 13                   |
| O (1–299)            | 488                  |
| O (19–139)           | 16                   |
| O (19–299)           | 242                  |

**Fig. 3.** Kinetics of hydrolysis of various λ O deletion mutant proteins by the ClpP/ClpX protease. Proteolysis assays were performed as described under “Materials and Methods.” The times listed under $t_{1/2}$ indicate the measured half-lives of the individual polypeptides in the proteolysis reaction. The amount of wild type or mutant λ O polypeptide present in each sample was calculated after densitometric analysis, using a Bio-Rad densitometer, of the relevant Coomassie Blue-stained band.

**Fig. 4.** Binding of ClpX to various λ O protein derivatives. The appropriate λ O protein derivative (50 μl, at a protein concentration of 10 μg/ml in phosphate-buffered saline buffer) or bovine serum albumin was fixed onto ELISA plate wells as described previously (18) and incubated for 1 h at room temperature. Increasing amounts of ClpX in buffer B were then added. Following a 30-min incubation at room temperature, the ELISA plates were washed once with buffer B and then incubated for an additional 2 h at room temperature. The amount of ClpX protein stably retained in each well was determined by use of a TMB peroxidase ELISA substrate kit (Bio-Rad). Absorbance at 490 nm was measured using a microplate reader. Each indicated absorbance value represents the average value obtained in four independent experiments.
ClpP and ClpX were not detected (data not shown). The amount of 1 mM ATP was measured using a microplate reader. In a control experiment, when TMB peroxidase EIA substrate kit (Bio-Rad). The absorbance at 490 nm were incubated for an additional 2 h at room temperature. The amount of ClpP/ClpX-dependent hydrolysis of O (19–139). Wild type or mutant O protein (50 μl, at a protein concentration of 10 μg/ml in phosphate-buffered saline buffer) was fixed onto the wells of an ELISA plate as described previously (18). Subsequently, increasing amounts of ClpP (in the presence of 100 ng of ClpX in buffer B) were added in the presence (open squares) or absence (closed squares) of 1 nm ATPγS. In a control experiment, increasing amounts of ClpP in the presence of ATP-γ-S (but in the absence of ClpX) were added (open circles, A). After a 30-min incubation at room temperature, the ELISA plates were washed once with buffer B and three times with phosphate-buffered saline supplemented with bovine serum albumin. Next, anti-ClpP serum (100 μl of a 1:8,000 dilution) was added, and the plates were incubated for an additional 2 h at room temperature. The amount of ClpP protein retained in individual wells was determined using a TMB peroxidase EIA substrate kit (Bio-Rad). The absorbance at 490 nm was measured using a microplate reader. In a control experiment, when bovine serum albumin instead of O was used, complexes containing ClpP were not detected (data not shown).

![Graph A](image1.png)

**Fig. 5. The binding of λ O to ClpP depends on the simultaneous presence of ClpX and ATP. A. λ O (1–299). B. λ O (1–139) or λ O (19–139) truncated protein, which binds ClpX. ATPγS was added in the presence (open squares) or absence (closed squares) of 1 nm ATPγS. In a control experiment, increasing amounts of ClpP in the presence of ATP-γ-S (but in the absence of ClpX) were added (open circles, A). After a 30-min incubation at room temperature, the ELISA plates were washed once with buffer B and three times with phosphate-buffered saline supplemented with bovine serum albumin. Next, anti-ClpP serum (100 μl of a 1:8,000 dilution) was added, and the plates were incubated for an additional 2 h at room temperature. The amount of ClpP protein retained in individual wells was determined using a TMB peroxidase EIA substrate kit (Bio-Rad). The absorbance at 490 nm was measured using a microplate reader. In a control experiment, when bovine serum albumin instead of O was used, complexes containing ClpP were not detected (data not shown).**

analogue ATPγS, which was previously shown to block the ClpP/ClpX-dependent hydrolysis of λ O (18). Using an ELISA assay, we determined the minimal requirements for ClpP to enter into a protein complex with O or O deletion mutant proteins (Fig. 5). Our results indicate that λ O does not enter into a complex with the ClpP protein unless both ClpX and ATPγS are present. The fact that the association of λ O with ClpP depends on ClpX suggests that at least in the presence of ATPγS, a λ O-ClpX-ClpP intermediate complex is formed. Interestingly the λ O (19–139) truncated protein, which binds moderately well to ClpX (Fig. 4) but is not hydrolyzed by ClpP/ClpX protease (Fig. 3), forms only a relatively weak complex with ClpP, even when both ClpX and ATPγS are present (Fig. 5). This result may indicate that the first 18 amino acids of λ O protein are required for efficient ClpX-dependent presentation of protein substrate to ClpP protease.

**DISCUSSION**

In a recent publication, Levchenko et al. (36) suggested that the ClpX protein recognizes specific sequences located at or near the C terminus of its substrate proteins. Their results indicated that such sequences may be displayed in a relatively disordered conformation. Experiments performed on MuA transposase, selected mutants of Mu repressor, and SsrA-tagged protein have implicated C-terminal residues of the substrate, mainly nonpolar amino acids, as being absolutely essential for ClpX recognition. As pointed out by Levchenko et al. (36), a second group of ClpX substrates is composed of proteins that contain predominantly polar amino acids near the C terminus, with arginine at the terminal position. In this respect, the λ O replication protein, whose 10 C-terminal residues (residues 290–299) are -NTDDWYGVL, apparently does not fit neatly into either of the known categories of ClpX substrates.

In this report, we present evidence that certain of the predictions for the first class of ClpX protease (36) are not applicable to λ O protein. Specifically, we showed that the N-terminal portion of λ O is more efficiently hydrolyzed by the ClpP/ClpX protease than is the O C-terminal region. The following experimental results support this conclusion: (i) the wild type λ O protein, O(1–299), is a much better substrate for ClpP/ClpX than either the O(19–299) or O(150–299) deletion mutant proteins, even though all three polypeptides share the same C-terminal sequence; (ii) three O deletion mutant proteins, O(1–162), O(1–139), and O(1–110), that each harbor a seemingly folded N-terminal domain, are rapidly hydrolyzed by ClpP/ClpX with kinetics similar to those for wild type λ O protein; (iii) the further removal of the first N-terminal 18 amino acids from λ O (1–110) or λ O (1–139) converts each to a form that is largely resistant to ClpP/ClpX-mediated proteolysis; (iv) the deletion of the first 18 amino acids at the N terminus of wild type λ O protein results in a partial stabilization of this protein against hydrolysis by the ClpP/ClpX protease; and (v) deletion of the 15 C-terminal residues of O(150–299) causes only a slight reduction in the rate of proteolysis by ClpP/ClpX.

This study also revealed that for most O deletion mutant proteins there is a close correlation between their relative affinity for ClpX and their rate of hydrolysis by the ClpP/ClpX protease. However, neither O(19–139) nor O(19–110) obeys this correlation. Both bind ClpX with moderate affinity, yet are nearly completely resistant to ClpP/ClpX-mediated proteolysis. We infer, therefore, that binding of ClpX to a potential substrate polypeptide is not sufficient to ensure hydrolysis of the polypeptide by the ATP-dependent ClpP/ClpX protease.

Although the N-terminal region of λ O plays the primary role in substrate recognition by ClpX, our investigations indicate that the C-terminal domain of O retains some sensitivity to the ClpP/ClpX protease. This finding suggests that a weaker ClpX recognition site(s) is located within the C-terminal region of the λ O polypeptide. Interestingly, O(19–299) and O(150–299), which possess different N-terminal sequences but identical C-terminal portions, are hydrolyzed by ClpP/ClpX protease with similar kinetics. This may imply, when the 18 amino acids at the N terminus are absent, that the C-terminal domain of λ O is more...
O determines the rate of ClpP/ClpX-dependent proteolysis. It is likely that relatively weak ClpX-binding sites are exposed on the surface of certain protein substrates of the ClpP/ClpX protease. In this regard, it is important to stress that such ClpX substrates as MuA transposase and Phd are hydrolyzed by the ClpP/ClpX protease much less efficiently than λ O.2,3 These ClpX substrates are hydrolyzed at rates roughly comparable with or even lower than that of the λ O(150–299). Thus, it is certainly possible that some proteins recognized by ClpX may possess multiple sequences or structural elements with varying degrees of affinity for ClpX.

Our results show that it is possible to identify proteins that bind to ClpX but are not hydrolyzed by the ClpP/ClpX protease. This implies that certain protein substrates that are hydrolyzed by the ClpP/ClpX protease, such as λ O, possess at least two structural elements that define its vulnerability to the protease. One element is presumably a ClpX recognition motif, whereas the other element apparently is composed, at least in part, of a specific sequence at the N terminus or C terminus of the polypeptide. The latter sequence may play a role in efficient delivery of a ClpX-bound polypeptide to the active site of the ClpP protease, perhaps by enabling efficient unfolding of the substrate. As shown in this paper, one of the ClpX recognition sites in λ O is located internally, somewhere within the 19–110-amino-acid fragment of O. The demonstration that ClpX recognition sites are not necessarily located at polypeptide termini agrees with a previous genetic analysis of the stationary phase sigma factor (σ^7), a study concluding that ClpX recognizes an internal amino acid sequence in this substrate protein (33). Regardless of which portion of a protein is primarily recognized by ClpX, it is likely that the final outcome, in term of proteolysis, will depend not only on the presence of an intrinsic high affinity ClpX recognition sequence but also on the availability of a special C- or N-terminal sequence to make contact with ClpX and/or ClpP during delivery of the substrate polypeptide to the ClpP catalytic subunit. This precise situation was encountered previously by Laachouch et al. (32). They fused the C-terminal 7 amino acids of the ClpP-sensitive Mu repressor vir3061 protein to the C termini of CcdA and CcdB proteins encoded by plasmid F. Fusion of this sequence to CcdB did not endow a sensitivity to ClpP/ClpX, whereas fusion of the same sequence to CcdA did engender a partial sensitivity to this ATP-dependent protease.

It was proposed previously that ClpX could participate in regulatory mechanisms that “decide” whether a particular protein substrate is either repaired or destroyed (1, 2, 12). This decision could be based on the affinity of ClpX for the various protein substrate is either repaired or destroyed (1, 2, 12). This decision could be based on the affinity of ClpX for the various
23. Jones, J. M., Welty, D. J., and Nakai, H. (1998) J. Biol. Chem. 273, 459–465
24. Nakai, H., and Kruklitis, R. (1995) J. Biol. Chem. 270, 19591–19598
25. Parsell, D. A., Kowal, A. S., and Lindquist, S. (1994a) J. Biol. Chem. 269, 4480–4487
26. Singh, S. K., and Maurizi, M. R. (1995) J. Biol. Chem. 270, 19591–19598
27. Seol, J. H., Woo, K. M., Kang, M. S., Ha, D. B., and Chung, C. H. (1995) Biochem. Biophys. Res. Commun. 217, 41–51
28. Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B., Singh, S. K., and Steven, A. C. (1995) J. Mol. Biol. 250, 587–594
29. Wang, J., Hartling, J. A., and Flanagan, J. M. (1997) Cell 91, 447–456
30. Grimaud, R., Kessel, M., Beuron, F., Steven, A. C., and Maurizi, M. R. (1998) J. Biol. Chem. 273, 12476–12481
31. Lehnherr, H., and Yarmolinsky, M. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3274–3277
32. Laachouch, J. E., Desmet, L., Geuskens, V., Grimaud, R., and Toussaint, A. (1996) EMBO J. 15, 437–444
33. Schweder, T., Lee, K. H., Lemovskaya, O., and Matin, A. (1996) J. Bacteriol. 178, 470–476
34. Frank, E. G., Ennis, D. G., Gonzalez, M., Levine, A. S., and Woodgate, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10291–10296
35. Gottesman, S., Wickner, S., and Maurizi, M. R. (1997) Genes Dev. 11, 815–823
36. Levenchenko, I., Smith, C. K., Walsh, N. P., Sauer, R. T., and Baker, T. A. (1997) Cell 91, 939–947
37. Gottesman, S., Roche, E., Zhou, Y., and Sauer, R. T. (1998) Genes Dev. 12, 1338–1347
38. Um, S.-J. (1993) Characterization of the Interaction of the Bacteriophage λ O Initiator Protein with the Viral Replication Origin. Ph.D. Dissertation, Johns Hopkins University
39. Karzai, A. W., and McMacken, R. (1996) J. Biol. Chem. 271, 11236–11246
40. Wold, M. S., Mallory, J. B., Roberts, J. D., LeBowitz, J. H., and McMacken, R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6176–6180
41. Russell, R., Jordan, R., and McMacken, R. (1998) Biochemistry 37, 596–607
42. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
43. Milman, G. (1987) Methods Enzymol. 153, 482–491
44. Roberts, J. D., and McMacken, R. (1983) Nucleic Acids Res. 11, 7435–7452
45. LeBowitz, J. H., Zylitz, M., Georgopoulos, C., and McMacken, R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3988–3992
46. LeBowitz, J. H., and McMacken, R. (1984) Nucleic Acids Res. 12, 3069–3088
47. Wyatt, W. W., and Inokuchi, H. (1974) Virology 58, 313–315
48. Lipinska, B., Podhajska, A., and Taylor, K. (1980) Biochem. Biophys. Res. Commun. 92, 120–126
49. Gottesman, S., Gottesman, M., Shaw, J. E., and Pearson, M. L. (1981) Cell 24, 225–233
50. McMacken, R., Wold, M. S., LeBowitz, J. H., Roberts, J. D., Mallory, J. B., Wilkinson, J. A. K., and Loehrlein, C. (1983) in Mechanisms of DNA Replication and Recombination (Cozzarelli, N. R., ed) pp. 819–848, A. R. Liss, Inc., New York
51. Bejarano, I., Klemes, Y., Schoulaker-Schwarz, R., and Engelberg-Kalka, H. (1993) J. Bacteriol. 175, 7720–7723
52. Wegrzyn, G., Pawlowicz, A., and Taylor, K. (1992) J. Mol. Biol. 226, 675–680
53. Zylitz, M., Liberek, K., Wawrznynow, A., and Georgopoulos, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15259–15263