ATP-dependent Degradation of CcdA by Lon Protease

EFFECTS OF SECONDARY STRUCTURE AND HETEROLOGOUS SUBUNIT INTERACTIONS*

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CcdA, the antidote protein of the ccd post-segregational killing system carried by the F plasmid, was degraded in vitro by purified Lon protease from Escherichia coli. CcdA had a low affinity for Lon (Km = 200 nm), and the peptide bond turnover number was ~10 min⁻¹. CcdA formed tight complexes with purified CcdB, the killer protein encoded in the ccd operon, and fluorescence and hydrodynamic measurements suggested that interaction with CcdB converted CcdA to a more compact conformation. CcdB prevented CcdA degradation by Lon and blocked the ability of CcdA to activate the ATPase activity of Lon, suggesting that Lon may recognize binding domains of proteins exposed when their partners are absent. Degradation of CcdA required ATP hydrolysis; however, CcdA41, consisting of the carboxyl-terminal 41 amino acids of CcdA and lacking the α-helical secondary structure present in CcdA, was degraded without ATP hydrolysis. Lon cleaved CcdA primarily between aliphatic and hydrophilic residues, and CcdA41 was cleaved at the same peptide bonds, indicating that ATP hydrolysis does not affect cleavage specificity. CcdA lost α-helical structure at elevated temperatures (Tm ~ 50 °C), and its degradation became independent of ATP hydrolysis at this temperature. ATP hydrolysis may be needed to disrupt interactions that stabilize the secondary structure of proteins allowing the disordered protein greater access to the proteolytic active sites.

Viability of bacterial cells harboring unit copy number plasmids is potentially compromised by the presence of plasmid-encoded gene products that are toxic to the cell. Cells carrying such plasmids survive because the plasmids encode, usually in the same operon with the toxin, a second gene product that acts as an antidote (reviewed in Refs. 1, 2). The antidote molecule is unstable and has a shorter half-life than the toxin; therefore, long-term survival of the cells requires the continuous production of the antidote. In cells that lose the plasmid, antidote concentrations decrease faster than those of the toxin, resulting in killing of the plasmid-cured cells. F plasmid contains three operons that function independently and with varying degrees of effectiveness as post-segregational killing systems (3–5). One such operon, ccd, plays a relatively minor role in post-segregational killing with intact F plasmid but, when present on a mini-F plasmid or when cloned on a plasmid with a heterologous replicon, results in killing of ~90% of plasmid-free segregants (3, 6, 7). ccd encodes CcdB, an 11-kDa protein that inhibits DNA gyrase (8–10), and CcdA, a 9-kDa protein that blocks the action of CcdB (6, 11).

CcdA is degraded in wild-type cells with a t1/2 ~ 30 min in the absence of CcdB and a t1/2 ~ 60 min in the presence of CcdB (12). Because CcdA is expressed in higher amounts than CcdB, it remains in excess of CcdB and neutralizes CcdB activity as long as the plasmid bearing ccd is maintained. Loss of the mini-F plasmid results in a decrease of CcdA over several generations leading to CcdB-mediated cell death. In lon mutants CcdA is degraded much more slowly (t1/2 ~ 120 min), and lon mutants survive loss of ccd-carrying plasmids (12). The half-life of CcdA is longer in the presence of CcdB (12), and autoregulation of the ccd operon requires the presence of both CcdA and CcdB (13, 14), both of which suggest that CcdA and CcdB interact with each other in vivo. The region of CcdA that interacts with CcdB resides within the carboxyl-terminal 41 amino acids, because a truncated form of CcdA with the first 31 amino acids deleted (CcdA41) also protects cells from the lethal effects of CcdB (15). CcdA and CcdB, either purified (16) or in cell extracts (17), bind to ccd operator DNA, and a complex of the two proteins was demonstrated by extraction from the ccd DNA and isolation by gel filtration (17).

Because CcdA and several other unstable proteins have longer half-lives in lon mutants, these proteins are probably degraded by Lon protease in vivo. Recognition of appropriate substrates by Lon protease and the control of Lon-dependent protein degradation in vivo, however, is still poorly understood. Both λ N protein (18) and a SulA fusion protein (19) are degraded in vitro by purified Lon protease, suggesting that Lon can directly recognize and degrade specific substrates. While molecular chaperones, such as DnaJ and DnaK, are required for some Lon-dependent degradation in vivo (20, 21), recent data suggest that this requirement applies only to proteins that aggregate rapidly in the absence of chaperones and thus become inaccessible to Lon protease (22). Degradation of CcdA, SulA, and λ N protein, for example, is independent of DnaJ in vivo (12). Because all of the known specific substrates for Lon protease are involved in macromolecular complexes in vivo, the distribution of the target proteins between free and complexed states and the kinetics of these interactions may be primary elements in control of their degradation (23). We have purified CcdA and have shown that, as expected, CcdA is degraded by
purified Lon protease. ATP hydrolysis is required for the initiating steps in Lon-dependent degradation of full-length CcdA, but the amino-terminally truncated CcdA11 is degraded by Lon without ATP hydrolysis. CcdB binds to both CcdA and CcdA11 and protects them from degradation by Lon, supporting the idea that subunit bonding domains may serve as recognition elements for Lon protease.

**EXPERIMENTAL PROCEDURES**

**Materials—**Polyacrylamide gradient gels and protein molecular weight standards were obtained from Bio-Rad. Lysyl endopeptidase (LysC) was obtained from Nanopure Chemical Industries, Ltd. Casein, nucleotides, and other phosphorylated compounds, phosphocreatine kinase, and staphylococcal endopeptidase Glu-C (V8 protease) were obtained from Sigma.

Preparation of CcdA, CcdB, and CcdA11—The CcdA protein was produced in CSH50 l:TiA1 carrying pULB2709 CcdA overproducing plasmid (9). Bacteria were grown in TB broth at 30 °C for 3 h, and expression of CcdA was induced by addition of 0.5 mM isopropyl-1-thio-

β-D-galactopyranoside. After 3 h induction, bacteria were harvested and stored at −70 °C until used. Frozen cells (25 g) were suspended in 100 ml of buffer B (Tris 50 mM, pH 7.5, EDTA 2 mM, and 10% glycerol) and broken in a French pressure cell at 20,000 psi. After centrifugation for 1 h at 16,000 rpm, the supernatant was treated with 0.4% polyethyl-

eneimine. Proteins (including CcdA) were extracted from the precipi-

tate in 100 ml of buffer B containing 500 mM NaCl. The extracted protein was precipitated with 40% saturated ammonium sulfate. After centrifugation, the pellet was suspended in 20 ml of buffer B and ap-
plied to a Mono-Q anion exchange column equilibrated with buffer B. Proteins were eluted using a linear gradient of NaCl. Fractions contain-
ing CcdA, which was eluted at 0.3 M isopropyl-

β-D-galactopyranoside. At 3 h induction, bacteria were harvested and stored at −70 °C until used. Frozen cells (25 g) were suspended in 100 ml of buffer B and CcdB were incubated for various times, and the reac-
tions were stopped by boiling in SDS sample buffer and heating or with an equal volume of 7 M guanidine HCl at room temperature. Cleavage of CcdA and CcdB by LysC and V8 protease was carried out in 50 mM Tris/HCl, pH 8.0, 1.0 mM EDTA, 0.2 mM KCl, and 10% glycerol.

**Enzymatic Assays—**Assays for casein degradation, ATPase activity, and peptidase activity were described previously (18). For degradation of CcdA by Lon, the protein (10–100 μg) was incubated in a final concentration of 50 mM Tris/HCl, pH 8.0, containing (in a typical assay) 4 mM ATP, 10 mM MgCl2, 1 mM diithiothreitol, 50 mM phosphocreatine, 2 μg of creatine kinase, and 1–4 μg of purified Lon protease. Degradation reactions were terminated after 1–2 h by mixing the solutions either with SDS-PAGE sample buffer and heating or with an equal volume of 7 M guanidine HCl at room temperature. Cleavage of CcdA and CcdB by LysC and V8 protease was carried out in 50 mM Tris/HCl, pH 8.0, 0.1 mM KCl, 1 mM EDTA, and 10% (v/v) glycerol. Aliquots of 30 μl containing 0.4 μg of protease and 8 μg of CcdA and/or CcdB were incubated for various times, and the reactions were stopped by boiling in SDS sample buffer (for SDS-PAGE analysis) or by adding 2 volumes of 7 M guanidine HCl (for reverse phase chromatography).

**Gel Filtration Analysis—**Analytical gel filtration was performed with a Superose 12 column using a buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 200 mM KCl, and 10% (v/v) glycerol. The flow rate was 0.3 ml/min. Molecular weight markers used were cytocerobulin (1,400,000 Da), myoglobin (17,000), ovalbumin (44,000), immunoglobulin G (160,000), and thyroglobulin (660,000).

**Analytical Ultracentrifugation—**Sedimentation equilibrium measurements of CcdA, CcdB, and the CcdA·CcdB complex were made in an Beckman XL-A ultracentrifuge with absorption optics. Sample columns of 160–200 μl in double sector cells with Kel-F center pieces and quartz windows were brought to equilibrium by centrifugation for 56 h at 190,000 rpm for CcdA, and for 72 h at 280,000 rpm for CcdB. Gradients at equilibrium were analyzed using nonlinear curve fitting programs.8 Solvent densities were measured with an Anton-Paar densitometer. Partial specific volumes for CcdA and CcdB were calculated from their amino acid compositions using the data for specific volumes of amino acids as described by Zamyannin (26). The partial specific volume of the CcdA·CcdB complex was calculated as the weight average of the two components assuming a molar ratio of 2 CcdB to 1 CcdA.

**Circular Dichroism—**Circular dichroism measurements were made with a Jasco 700 Spectropolarimeter. Samples of proteins were prepared in 10 mM Tris/HCl, pH 8.0, and diluted to give an absorbance at 280 nm of 0.2–0.3 (1-cm path length). CD spectra were recorded at constant temperature in water-jacketed cylindrical cells with 0.2-cm path length at a scan rate of 1 nm/s; four averages were taken for each spectrum. Melting curves were recorded at 222 nm by increasing the temperature at 1 °C/min from 10 to 80 °C.

**Polyacrylamide Gel Electrophoresis—**Polyacrylamide gel electrophoresis in the presence of SDS was performed using the Laemmli buffer system with 15% acrylamide gels prepared in this laboratory or 4–20% gradient gels obtained from Bio-Rad. Native gel electrophoresis was done using the Bio-Rad gradient gels but SDS was omitted.

**Cross-linking—**For chemical cross-linking of CcdA and CcdB, proteins were dialyzed against 100 mM triethylamine, pH 9.0, and incubated with 25 mM (disuccinimidyl) suberate for 2–60 min. Protein concentrations were varied from 10 to 100 μg/ml. Reactions were quenched by addition of Tris/HCl, pH 8.0, and NaCl, both to a final concentration of 0.2 M. Aliquots of the reaction mixture were then boiled in SDS, separated on SDS-Tricine gels, and stained.

**Reverse Phase Chromatography—**Protein and peptide sample were prepared by mixing the solutions with an equal or greater volume of guanidine HCl so that the final concentration was 3.7–6 M guanidine HCl. Samples were applied to a Vydac C-18 protein/peptide column (7 mm × 25 cm) equilibrated with aqueous 0.05% trifluoroacetic acid, and peptides were eluted with a linear gradient of acetonitrile containing 0.05% trifluoroacetic acid.

**Pep tide and Protein Sequencing—**Peptide and protein fractions eluted from reverse phase columns, in acetoni trile/water/trifluoroacetic acid mixtures, were dried onto glass fiber filters treated with Biotrene, and the sequence from the amino terminus was obtained by Edman degradation using an automated Applied Biosystems 476A peptide sequencer according to the manufacturer’s recommendations. Peptides were usually sequenced through to the end.

**Mass Spectrometry—**Peptide fractions obtained from reverse phase columns were analyzed by matrix assisted laser desorption mass spectrometry (MALDI) with a MALDI III-TOF (time of flight) analyzer (Shimadzu) operating at an accelerating voltage of 22 kV. For each sample, 0.7 μl was mixed with 1 μl of matrix compound (a 50/50 (v/v) mixture of acetonitrile and 10 mg/ml a-cyano-4-hydroxycinnamic acid in 1% aqueous trifluoroacetic acid). Millitin-H+ (M, 2848.5) and one peak from the matrix (M, 3794.4) were used for external calibration (27). To confirm the results obtained by MALDI, peptide fractions were concentrated and analyzed by fast atom bombardment (FAB) mass spectrometry. FAB spectra were obtained on a Jet SX102 mass spectrometer (Jebula Inc.) operating at an accelerating voltage of 10 kV. Samples were mixed with a matrix compound and then desorbed using 6 keV xenon atoms (28).

**RESULTS**

**Properties of CcdA and CcdB—**The DNA sequence of the ccdA gene predicts a protein of 72 amino acid residues (M9, 3787). CcdA was overexpressed from a multicopy plasmid and purified to essential homogeneity on the basis of its apparent size by SDS-PAGE analysis. Identity of CcdA was confirmed by amino-terminal sequencing of the first 20 amino acid residues. The aromatic amino acid content of CcdA determined by decomposition of the UV absorbance spectrum of the protein denatured in guanidine was identical to that predicted from the DNA sequence, and the extinction coefficient at 280 nm of CcdA was calculated to be 1.6 (mg/ml)−1. Some preparations of CcdA had a small amount (<10%) of CcdA truncated by several

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Tricine, N-(2-hydroxy-1,1-bishydroxymethyl)glycine; AMPNNP, adenosine 5′-p′-γ-iminophosphoribosyl. 2 Program written by A. Minton, NIDDK, Bethesda, MD. These programs can be downloaded from the following site: http://bbri-www.eri.harvard.edu/RASMB/rasmb.html.
Equilibrium ultracentrifugation of CcdA gave a molecular weight of 17 ± 1 × 10^3 (Table I) suggesting that the native form of CcdA is a dimer. Cross-linking with (disuccimidyl) suberate and analysis by SDS-PAGE showed a major species (>50%) with a Mr of 18,000 as well as products of higher molecular weight (data not shown). The Mr of 18,000 species predominated when cross-linking was done for short periods at low protein concentrations as expected if the CcdA is a dimer. CcdA eluted in a single sharp symmetrical peak on gel filtration columns under nondenaturing conditions (Fig. 1A) and appeared as a single band after native gel electrophoresis (Fig. 2, A and C). The mobility of CcdA relative to standard proteins on gel filtration columns gave an estimated Mr of 25,000 (Fig. 1B), significantly higher than expected for a homodimer. This anomalously high Mr probably reflects an extended conformation and consequently larger Stoke’s radius and faster mobility on gel filtration columns. The intrinsic fluorescence of CcdA under nondenaturing conditions is red-shifted (see below), indicating solvent-exposed tryptophan residues consistent with an extended or partially unfolded conformation for CcdA. The mutant protein, CcdA41, which possesses only the carboxyl-terminal 41 amino acids of CcdA, eluted in an asymmetrical peak from a gel filtration column (data not shown). The predominant form had a mobility consistent with a dimer (Mr ~9,000), and the trailing species was probably the dissociated monomer.

The ccdB gene encodes a protein with 101 amino acids (Mr 11,706). CcdB was purified to homogeneity (24), and its identity was confirmed by amino-terminal sequencing and analysis of the aromatic amino acid content by deconvolution of the UV absorbance spectrum. The extinction coefficient at 280 nm of the aromatic amino acid content by deconvolution of the UV absorbance spectrum. The extinction coefficient at 280 nm of CcdB was 1.4 (mg/ml)^-1 (24). Cross-linking of CcdB produced a dimeric species as determined by SDS-PAGE (data not shown). On gel filtration under native conditions, the major species of CcdB behaved as a dimer with apparent Mr of 22,000 (Fig. 1, A and B); a small amount of tetrameric CcdB (Mr 44,000) was also found.

Complex of CcdA and CcdB—CcdA and CcdB interact to form a stable complex that can be isolated by gel filtration (Fig. 1A) or native gel electrophoresis (Fig. 2, A and C). By gel filtration the complex had an estimated Mr of 60,000, close to that expected for a complex of a tetramer of CcdB with a dimer of CcdA (Fig. 1, A and B). The presence of both CcdA and CcdB in the complex was confirmed by SDS-PAGE (Fig. 2A). Equilibrium sedimentation of the CcdA:CcdB complex isolated by gel filtration gave a molecular weight of 58,000 (Table I). By native gel electrophoresis, the CcdA:CcdB complex had a mobility between that of CcdA and CcdB alone (Fig. 2, A and C). Fig. 2C shows that, as the amount of CcdB was increased, the band in the CcdA position disappeared and a somewhat diffuse band of lower mobility appeared. The diffuseness of the band, which represents a species with equimolar ratios of CcdA and CcdB, is caused by the insolubility of this form of the complex (data not shown). Further additions of CcdB resulted in a sharper band representing a soluble complex. Free CcdB was not observed until the molar ratio of CcdB to CcdA was greater than 2:1, consistent with stoichiometry of the complex predicted by gel filtration. Studies done in vitro showing that the truncated mutant protein, CcdA41, can neutralize the activity of CcdB suggest that CcdA41 also forms a complex with CcdB (15). As seen for CcdA, the complex of CcdA41 and CcdB sep-
arated by native gel electrophoresis had a mobility intermediate between those of CcdA41 and CcdB. Titration experiments showed that free CcdA41 remained until CcdB was added in a 2-fold molar excess, suggesting that two dimers of CcdB bind to a dimer of CcdA41 (Fig. 2B). On some gels, a complex between CcdB and a lower molecular weight form of CcdA41 (which appeared in older stocks of the protein) was also evident (data not shown).

ATP-dependent Degradation of CcdA by Purified Lon Protease—CcdA was degraded by Lon protease in the presence of ATP; however, no degradation was observed when a nonhydrolyzable analog of ATP, AMPPNP, was used (Fig. 3A). The presence of an ATP-regenerating system extended the reaction time and allowed more of the CcdA to be degraded (Fig. 3A, fifth versus third lane). CcdA41 was also degraded by Lon, but in this case similar degradation rates were observed with either ATP or AMPPNP (Fig. 3B and data not shown).

Effects of Secondary Structure on Degradation of CcdA and CcdA41—The differences in nucleotide requirement for degradation of CcdA and CcdA41 prompted us to examine the secondary structure of both proteins. Fig. 4 shows the circular dichroism spectra for CcdA and CcdA41 recorded at 20 °C. CcdA had negative peaks at 222 and 210 nm, characteristic of α-helices, and the calculated α-helical content of 55% agreed well with that predicted from the amino acid sequence (16). The secondary structure predictions indicate that the α-helices in CcdA should reside in the carboxyl-terminal portion, in the region corresponding to CcdA41. Purified CcdA41, however, contained almost no α-helical or β-sheet structure. Lowering the temperature to 5 °C produced only a modest increase of α-helical structure (data not shown). These data suggested that the requirement for ATP hydrolysis only for the degradation of CcdA reflects the need for work in disrupting the secondary structure of CcdA but not CcdA41.

Circular dichroism measurements indicated that CcdA underwent a reversible thermal unfolding with a melting point of 50.5 °C (Fig. 4). At 80 °C, CcdA had lost identifiable secondary structure, but the α-helical content was completely restored when the temperature was lowered to 20 °C (Fig. 4). To see if thermally disrupting the secondary structure of CcdA would affect either its susceptibility to degradation or the require-
Structural Effects on ATP-dependent Degradation by Lon

Fig. 4. Circular dichroism of CcdA and CcdA41. Spectra of protein samples in 10 mM Tris/HCl, pH 7.5, were recorded with a Jasco J700 spectropolarimeter at the temperatures indicated. The temperature was raised at a rate of 1 °C/min and lowered to room temperature within 10 min. Spectra of CcdA (—) and CcdA41 (⋯⋯) at 20 °C and of CcdA at 80 °C (⋯⋯⋯). The spectrum of CcdA after returning the temperature to 20 °C was identical to that before raising the temperature, and the spectrum of CcdA41 showed very little change when the temperature was raised (data not shown). Inset, the circular dichroism of CcdA was measured at 222 nm as the temperature was raised from 20 to 80 °C.

Nucleotide Dependence of CcdA and CcdA41 Degradation—The $K_a$ for ATP, measured in the presence of an ATP-regenerating system, was about 5 μM (Table III). This value is somewhat lower than the values reported previously but is consistent with the apparent $K_a$ for binding of ATP to the “low affinity” sites on Lon reported by Menon and Goldberg (29). CcdA41 degradation was activated by submicromolar concentrations of AMPPNP with an apparent $K_a$ of 0.2 μM (Table III). The actual $K_a$ for AMPPNP could be even lower than estimated, because the $K_a$ was in the same range as the concentration of Lon needed to observe significant cleavage of CcdA41 under these assay conditions. These data are consistent with the proposed role for very tight binding of ATP or AMPPNP to the “high affinity” sites in activating peptidase activity of Lon (29).

Degradation of CcdA41 was also activated by CTP and pyrophosphate and to a lesser extent by UTP and GTP (data not shown), as has been seen for Lon-dependent cleavage of small peptide substrates (30). Our data show that allosteric activation of Lon by nucleotides or other polyphosphate binding is sufficient to open the active site to moderately long polypeptides (≤41 amino acids) provided they are unfolded.

Kinetics of CcdA and CcdA41 Cleavage by Lon—The peptides generated by digestion of CcdA and CcdA41 by Lon were separated by reverse phase chromatography. Casein degradation was measured by the appearance of acid-soluble peptides.

Proteins were preincubated for 5 min at the indicated temperatures in assay solutions with either 4 mM ATP or 1 mM AMPPNP. Lon was added to initiate the degradation reaction which was carried out for 30 min. Degradation of CcdA and CcdA41 was measured by the appearance of peptide products separated by reverse phase chromatography. Casein degradation was measured by the appearance of acid-soluble peptides.
saturating amounts (Fig. 6). The \( V_{max} \) versus \( 1/K_m \) plot for CcdA41 was concave upwards, suggesting possible positive cooperativity in substrate binding. The \( K_m \) for CcdA41 was around 250 \( \mu M \) (Table III), and given the close agreement of the CcdA data with the CcdA41 data at lower substrate concentrations, it is likely that the \( K_m \) for CcdA is also \( \geq 200 \mu M \) (Fig. 6 and Table III). The extrapolated value of the \( V_{max} \) was about 13 mg of CcdA degraded per h/mg of Lon (Fig. 6). Because the average CcdA molecule was cleaved five times (see below), the turnover number for CcdA degradation was estimated to be about 10 min\(^{-1}\), calculated as peptide bonds cleaved per min per active site of Lon protease (assuming one active site per subunit). The CcdA turnover rate is higher than that for \( \alpha \)-casein (2 min\(^{-1}\)) (30) but lower than that for \( \lambda \) N protein (60 min\(^{-1}\)) (18). The turnover number for CcdA41 degraded in the presence of AMPPNP was about 5 min\(^{-1}\). CcdA and CcdA41 were also tested as inhibitors of \( \alpha \)-casein degradation. The \( K_i \) value calculated assuming competitive inhibition was \( \geq 80 \mu M \) for both proteins, which also indicates rather low affinity between Lon and either CcdA or CcdA41.

Many protein substrates activate both hydrolysis of ATP and cleavage of small fluorogenic peptides by Lon (31). The latter activity is believed to reflect an allosteric binding site for protein substrates that allows access to the active site for small peptides and unfolded regions of the protein substrate. CcdA activated the ATPase activity of Lon (Table IV). The \( K_m \) for activation was lower than the apparent \( K_m \) and \( K_i \) values (data not shown), which suggests that, as with other Lon substrates, CcdA interacts with Lon at an allosteric site.

Peptide Bonds Cleaved in CcdA and CcdA41—The peptide products from CcdA and CcdA41 degradation were identified after isolation by reverse phase chromatography, and the deduced sites of cleavage in the two proteins are shown in Fig. 7A. The sites cleaved in CcdA and CcdA41 were similar in nature to those cleaved in A N protein, insulin B chain, and glucagon (18). Although there is no absolute sequence specificity near the site of cleavage, the junctions between relatively hydrophobic and hydrophilic regions tend to be cleaved, albeit somewhat imprecisely. In CcdA, Ala is found at P1 (the first residue on the amino side of the cleavage site) four times and Glu is found at P1' (the first residue on the carboxyl side of the cleavage site) two times. Where two or more hydrophobic residues precede a hydrophilic residue, cleavage occurs between the hydrophobic residues (e.g. between Leu\(^{16}\)-Leu\(^{17}\) and Phe\(^{58}\)-Thr\(^{59}\)). When the hydrophilic residues precede more than one charged or hydrophilic residue, cleavage can occur at several sites including between the hydrophilic residues. This alternate site cleavage (three of five sites in LLKAYD and four of five sites in LVSTTM) suggests that the active site cavity is large enough or perhaps malleable enough to allow slippage in positioning of the polypeptide chain for cleavage. As has been observed before, some sites with seemingly preferred amino acids (e.g. Ala\(^{53}\)-Glu\(^{54}\)) are not cleaved at all, possibly reflecting important interactions (either favorable or unfavorable) involving residues some distance from the cleavage site or indicative of a
role for the local conformation of the polypeptide chain in positioning particular peptide bonds for cleavage.

*CdcB Protects CcdA from Degradation by Lon*—The presence of CcdB in excess over CcdA or CcdA41 prevented their degradation by Lon (Fig. 3A and B). CcdB had no effect on degradation of α-casein by Lon (data not shown) or on Lon’s ATPase activity (Table IV). Complete protection of CcdA from Lon required CcdB in amounts equal to or greater than CcdA (data not shown). When the CcdB complex was present in limiting amounts, only the uncomplexed portion of CcdA was degraded, and the pattern of degradation judged by the peptide profile was identical to that obtained with CcdA alone (data not shown). CcdB blocked activation of Lon ATPase activity by CcdA (Table IV), which suggests that CcdB can not interact with Lon when CcdA and CcdB are in a complex. Thus, CcdB protects CcdA probably by masking or making inaccessible those regions of CcdA normally recognized by Lon.

CcdB also partially protects CcdA from degradation by proteases such as LysC and V8 protease. Fig. 7A also shows the cleavage sites in CcdA incubated with these proteases in the absence and in the presence of CcdB. LysC cuts CcdA after all three lysine residues, two near the amino terminus and one about two-thirds of the way through the protein, and V8 protease cuts at three sites, all in the carboxyl-terminal half of CcdA (which will be referred to as the A41 domain). CcdB protects the A41 domain from cleavage by both proteases, but the cleavage sites for lysyl endopeptidase in the amino-terminal portion of CcdA are not protected at all by CcdB. These data are consistent with earlier data (Fig. 2) showing that the mutant CcdA41 binds to CcdB and indicate that at least a portion of the amino-terminal domain of CcdA and CcdB remains exposed in the CcdA-CcdB complex. This exposed amino-terminal portion of CcdA, however, cannot be targeted by Lon.

Fig. 7B shows the sites in CcdB cleaved by LysC and indicates the region of CcdB protected by CcdA. Sites near the amino terminus and the carboxyl terminus remain accessible to LysC when CcdB is complexed with CcdA suggesting that those regions of CcdB are not involved in either CcdB-CcdB or CcdB-CcdA binding domains.

*CcdA May Become More Compact When Complexed with CcdB*—As indicated in Table I, CcdA showed properties of a protein with an extended conformation, whereas the CcdA-CcdB complex did not. Thus, binding of CcdB appears to convert CcdA to a more compact or globular state. Changes in CcdA fluorescence also suggest a conformational change in CcdA upon binding CcdB. The intrinsic fluorescence of CcdA had an emission maximum at 346 nm, suggesting that both tryptophan residues are highly solvent-exposed (Fig. 8). Addition of CcdB results in a dramatic blue shift in the fluorescence emission which had a maximum near 330 nm (Fig. 8), indicating that both tryptophan residues are buried in the complex. The intrinsic fluorescence of CcdA41 was similar to that of CcdA and was blue-shifted to a similar extent upon binding of CcdB (data not shown). Burial of the tryptophan residues could indicate that those residues form part of the bonding domain between CcdA and CcdB or that CcdB binding induces a conformational change in CcdA that moves the tryptophan residues away from the solvent into closer contact with other residues in the terminal α-helix. Because CcdA which lacked the carboxyl-terminal tryptophan nonetheless appeared to form stable complexes with CcdB (data not shown), it seems unlikely that the C-terminal tryptophan residue is an integral part of the bonding interface between these proteins.

**DISCUSSION**

Selection of appropriate substrates by the ATP-dependent proteases in cells is a critical step in regulatory degradation. Selection entails both an active and a passive element. The active element is a specific motif (a single residue, a contiguous or interrupted sequence, or a general chemical or structural feature) that is recognized by the protease or, as in the case in eukaryotic cells, by a component of a system that presents substrates to the protease (for reviews see Refs. 32, 33). The passive element includes factors that affect the accessibility of the potential substrates, such as temporal and spatial compartmentalization, ligand binding, and macromolecular interactions (23). In this paper, we have shown that formation of a bimolecular complex between CcdA and CcdB prevents CcdA degradation either by altering the conformation of CcdA so it is no longer recognized by Lon protease or by sterically hindering access to the recognition site. Our results are consistent with in vivo studies showing that CcdA has an extended half-life in the presence of CcdB (12).

CcdA is degraded in vivo and is more stable in lon mutants (12). Our data demonstrate that purified CcdA is degraded in vitro by purified Lon protease, thus confirming the ability of Lon protease to directly recognize and degrade specific substrates without the obligatory participation of other proteins. Degradation of CcdA was dependent on ATP hydrolysis, and the turnover rate for CcdA was comparable with that observed...
for other protein substrates in vitro. Interestingly, the relative degradation rates of CcdA and λ N protein (18) in vitro are proportional to their turnover rates in vivo; λ N protein appears to be degraded about 5–10 times faster in both conditions. The peptide bonds cleaved in CcdA and CcdA41 had the general property observed with other Lon substrates. Cleavage came after a hydrophobic residue in 9 of 12 sites and before a hydrophilic residue in 9 of 12 sites. The exceptions always occurred at complex sites where a sequence with several hydrophobic residues was followed by several hydrophilic residues, a condition that appears to allow slipping or variable positioning of the polypeptide in the active site. As seen previously for other substrates in vitro (e.g. λ N protein (18)), CcdA had a high $K_m$ as a substrate for Lon. Thus it appears that physiological substrates of Lon may not possess unique high affinity motifs by which they are recognized.

The function or functions of ATP hydrolysis in Lon-dependent proteolysis have not been defined, although several models have been proposed (23, 34, 35). One can identify the following steps in the degradation reaction: 1) interaction between the protein substrate and Lon; 2) unfolding or other conformational change in the substrate; 3) translocation of some portion of the substrate into the active site; 4) cleavage of an initial but not necessarily unique peptide bond; 5) continued translocation of the remaining portions of the polypeptide into the active site; and 6) continued cleavage of the remaining susceptible peptide bonds in the substrate. Both CcdA and CcdA41 are recognized by Lon, degraded at comparable rates, and cleaved at the same peptide bonds. In one case ATP hydrolysis is required and in the other it is not. Peptidase release from CcdA41 in the presence of AMP/PNP is essentially the same as in the presence of ATP. Thus, the latter steps in the degradation cycle involving positioning of the polypeptide in the active site of Lon and enabling multiple cuts in some region of the protein molecule are not energy-dependent steps. Because no cleavage of intact CcdA is observed without ATP hydrolysis, the energy-dependent step in CcdA degradation must precede the initial cleavage reaction.

The subsequent reactions that are common to CcdA41 appear to proceed without energy consumption. Thus, for these small proteins at least, rate and specificity of peptide bond cleavage is independent of ATP hydrolysis once an initial energy barrier is overcome. For larger proteins there might be a series of such energy barriers representing different secondary or tertiary structural features.

Secondary structure predictions suggest that the domain in common between CcdA and CcdA41 (the A41 domain) contains two or three helices and that the amino-terminal domain contains two strands of β-sheet separated by a loop and/or small helix (Fig. 9). The differences in stability of their secondary structures may be responsible for the difference in energy requirement for degradation of CcdA and CcdA41. Since CcdA41 and CcdA have similar affinities for Lon, it is unlikely that unfolding of CcdA simply unmasks a high affinity recognition site for Lon. The folded secondary structure of CcdA may hinder access to the proteolytic active sites. ATP hydrolysis could be involved either in directly disrupting secondary structure or in unfolding tertiary structures that stabilize the secondary structures. Other studies have shown a general correspondence between thermal stability of proteins in vitro and their metabolic half-lives (36, 37). For T4 lysozyme mutants, degradation in vivo was faster for variant forms with lower thermal melting temperatures, except when the mutant proteins had a tendency to form inclusion bodies that rendered them inaccessible to intracellular proteases (38). Our data offer a refinement on those observations, suggesting that thermal stability determines not only chemical instability but also the energy required for degradation of the unstable proteins. Proteins that do not tend to aggregate in inclusion bodies or do not have stable, difficult to unfold domains may be degraded in vivo with minimal energy expenditure.

We propose that the barrier to degradation in CcdA involves interaction between the A41 domain and the amino-terminal domain, which is absent in CcdA41. CcdA41 may have sufficient flexibility to bind to Lon and to enter efficiently the proteolytic active sites, but in CcdA that flexibility is limited by interaction with the amino-terminal domain. ATP hydrolysis would be needed to overcome favorable interactions between the two domains. The loop between the β-sheets in CcdA contains a lysine residue that is accessible to lysyl endopeptidase even when CcdA is in a complex with CcdB, indicating that CcdB does not impinge closely on the β-sheet region of CcdA. CcdB may bind to a face of one or more of the helices in the A41 domain, which might correspond to the region of CcdA that is recognized by Lon protease, since CcdB blocks interaction between CcdA and Lon. By strengthening the interaction between the A41 and amino-terminal domains of CcdA, CcdB would hinder energy-dependent disruption of CcdA structure and offer further protection from degradation by Lon.

Lon, a multimer of identical subunits, represents a major class of ATP-dependent proteases that have the ATPase domain and proteolytic domain encoded within a single polypeptide chain. The other major classes, which includes the Clp proteases and the eukaryotic 26 S proteasome, are hetero-oligomeric complexes of independently expressed ATPase and proteolytic subunits. Evidence obtained with the *Escherichia coli* ClpAP protease indicates that the ATPase component has a chaperone-like protein remodeling activity (38). Although this chaperone activity may function to restore native structure to unfolded proteins, it is probable that its major role is to promote further unfolding of proteins with abnormal structures to enable them to be degraded by the associated proteolytic components. Data in this paper can be interpreted to support a similar unfolding activity for the ATPase domain of Lon prote-
ae. Encoding of both activities within the same protein might suggest that the Lon chaperone activity is dedicated exclusively to unfolding of proteins for degradation. We propose that protein unfolding enzymes that carry out this grim chaperone activity should be referred to as charonins.

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Fig. 9. The predicted secondary structure was calculated from the sequence of CcdA using the Chou and Fasman algorithm provided by the GCG sequence analysis package (39, 40). The arrows indicate the cleavage sites by Lon protease, lysyl endopeptidase, and V8 protease, as described in the legend to Fig. 7. Secondary structure features are indicated by bands for β-sheets, ribbons for α-helices, and thin lines for coiled or unstructured regions.