Activity and Specificity of Escherichia coli ClpAP Protease in Cleaving Model Peptide Substrates*

(Received for publication, March 22, 1994, and in revised form, April 28, 1994)

Mark W. Thompson and Michael R. Maurizi‡
From the Laboratory of Cell Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892

Escherichia coli ClpAP protease is an ATP-dependent protease composed of the proteolytic component ClpP and a regulatory ATPase, ClpA. ClpAP protease degraded a variety of peptide bonds in protein and peptide substrates at a slow rate ($k_{cat} \approx 30$ min$^{-1}$/subunit of ClpP), but showed very high activity ($k_{cat} \geq 800$ min$^{-1}$) for a synthetic peptide composed of the first 19 amino acids of ClpP, MSYSGERDNFAPHMAVLVP, referred to as the peptidase. The peptidase was not degraded by ClpP alone, but was degraded in the presence of ClpA and ClpP. Degradation was activated by nonhydrolyzable analogs of ATP, indicating that nucleotide-promoted interaction between ClpA and ClpP is sufficient to activate ClpP for peptidase cleavage. The peptidase, as well as truncated forms lacking either the first 9 or the last 3 amino acids, was cleaved at the same Met-Ala bond at which autoprocessing occurs in vivo. No hydrolysis of FAPMHLVVP derivatives was observed when Met was replaced by Glu, Lys, Ser, Tyr, Ile, and p-Met, but cleavage at the same position did occur with Leu or Trp substitutions. A peptide composed of a tandem repeat of FAPMHLVVP was cleaved between both Met-Ala bonds ($k_{cat} \approx 39$ min$^{-1}$). Peptidases inhibited degradation of a casein by competition for a binding site on ClpA, and they stimulated the basal ATPase activity of ClpA in the absence of ClpP. Peptides and protein substrates interact at an allosteric site on ClpA, which appears to be the site at which specific substrates are recognized by the Clp protease.

The selectivity of protein turnover in vivo is a complex function of the sequences and structures of intracellular proteins and the specificity and activity of intracellular proteases. The two known major ATP-dependent proteases of Escherichia coli, Lon and ClpAP, degrade different specific protein substrates (1-3), although both contribute to degradation of abnormal canavanyl proteins (4). The mechanism by which Lon protease or ClpAP protease recognizes particular proteins is not known. Binding of proteins to Lon protease can activate ATPase activity independently of protein degradation (5) and activates peptidase activity against short peptides (6), suggesting that Lon may possess a separate protein recognition site in addition to the proteolytic active site.

The ClpAP protease of E. coli is a complex, multicomponent ATP-dependent proteolytic system. The first components of the system to be isolated were ClpP, a dodecameric serine protease (7-9), and ClpA, an ATPase that forms a complex with ClpP and promotes ClpP-dependent cleavage of proteins in vitro (7, 9, 10). Studies with mutants indicate that ClpP and ClpA are both required for degradation of specific β-galactosidase fusion proteins and thus form an active protease in vivo (3, 11, 12). A second ATPase, ClpX, that activates ClpP for degradation of λ O protein in vivo and in vitro has recently been described (2, 13). Mutants lacking either ClpA or ClpX are defective for degradation of their respective substrates, but not each others, suggesting that the ATPase components play a major role in the recognition and degradation of specific protein substrates.

Sequence comparisons have led to the identification of other E. coli protein members of the Clp family (14). ClpP is a heat shock protein that affects thermotolerance of E. coli (16) and has ATPase activity that is activated by proteins in vitro (17, 18), and ClpY was identified as an open reading frame that is highly homologous to ClpX (2). No evidence has yet been reported that ClpB or ClpY can activate proteolysis by ClpP or another protease. ClpP occurs in an operon with another protease, ClpY, which appears to be related to subunits of the eukaryotic proteasome or multicatalytic proteinase.1 It remains to be shown if all of the different Clp ATPases direct specific substrates to ClpP or other proteases or carry out chaperone functions unrelated to degradation (19).

This paper and the accompanying paper (20) address questions about the specificity of the proteolytic activity of ClpAP and the role played by ATP in activating proteolysis. Dimeric ClpA and dodecameric ClpP are purified separately and do not associate with each other in the absence of nucleotide (7, 9, 10). ATP promotes formation of a ClpA hexamer, which associates tightly with ClpP to form the ClpAP protease (10). ClpP alone can degrade a short fluorogenic peptide, N-succinyl-Leu-Tyr 7-amido-4-methylcoumarin (21), but degradation of proteins by ClpP requires ClpA as well as ATP (7, 22). No nucleotides other than ATP and dATP can activate protein degradation by ClpAP, and hydrolysis of the ATP is required. In this paper, we show that a specific peptide substrate of ClpAP interacts at an allosteric site on ClpA where it competes with protein substrates and that nucleotide-promoted interaction between ClpA and ClpP is sufficient to activate ClpP to degrade the peptide at rates faster than those previously reported for substrates of other ATP-dependent proteases.

EXPERIMENTAL PROCEDURES

Materials—Tris and crystalline phenol were purchased from Life Technologies, Inc. ATP, AMP-PNP,2 ATP$\gamma$S, Q-Sepharose, S-Sepharose, and N-succinyl-Leu-Tyr 7-amido-4-methylcoumarin were obtained from Sigma. Polyethyleneimine was from ICN. Mono Q and Mono S were from Pharmacia Biotech Inc. [3H]Formaldehyde from DuPont NEN. N-Methylpyrrolidine, piperidine, N,N-diisopropylethyamine, and dicyclohexylcarbodiimide were purchased from Pierce. N,N-Dimethylformamide, pyrrolidine, and N,N-diisopropylethylamine were purchased from Aldrich. HPLC, high pressure liquid chromatography.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: NCI, National Institutes of Health, Bldg. 37, Rm. 1B07, Bethesda, MD 20892. Tel.: 301-496-7961; Fax: 301-402-0450.
‡ Current address: Laboratory of Molecular Medicine, Harvard University, Cambridge, MA 02138.

2 The abbreviations used are: AMP-PNP, adenosine 5'-O-(3-thiotriphosphate); ATP$\gamma$S, adenosine 5'-O-(3-thiotriphosphate); Fmoc, N-N-diisopropylmethoxy carbonyl; HPLC, high pressure liquid chromatography.
Calculated from the integrated areas of the 260 nm absorbance peaks using empirically determined relative absorbances of 2 for tryosine and 4 for tryptophan.

**Synthesis and Purification of Peptide Substrates**—The synthesis of ClpP propeptides and pentapeptide amides was done using FastMoc™ methodology (24) on an Applied Biosystems Model 431A peptide synthesizer. The synthesizer was programmed with FastMoc™ methodology using SynthAssist™ software (Applied Biosystems). Solid-phase synthesis of ClpP propeptides was done on a 0.25- or 0.1-mm scale using hydroxymethylphenoxymethyl resin to provide a carboxy-terminally blocked coumarin amide. The pentapeptide amides were synthesized using Rink™ amide (4-[(2′,4′-dimethoxyphenyl)-N-monomethyl-phenyl] -penoxyl) resin. The peptide was cleaved from the resin in a 10-ml reaction mixture containing 82.5% trifluoroacetic acid, 5% phenol, 5% 2,5-thiophenol, and 2.5% ethanedithiol as described by King et al. (34). The peptide was purified by gel filtration using Sephadex G-25 with 5% acetic acid as the solvent and further purified by C, reverse-phase HPLC using an aqueous acetonitrile gradient in 0.06% trifluoroacetic acid. Purified peptides were lyophilized and stored at -70 °C. **Amino Acid Analysis**—Peptides were hydrolyzed in 6 N HCl for 45 min at 110 °C, and the resultant amino acids were derivatized as described (25). Phenylthiocarbamyl-derivated amino acids were separated at 51 °C on a C, reversed-phase column (14 × 0.6 cm) using a gradient composed of solvent A (0.14 M sodium acetate and 0.05% [v/v] triethylamine adjusted to pH 6.35 with glacial acetic acid) and solvent B (60% aqueous acetonitrile in water).

**RESULTS**

Small Hydrophobic Pentapeptides Are Cleaved by ClpP and Are Effective Inhibitors of ClpP Peptidase and ClpAP Protease Activity—The fluorogenic peptide N-succinyl-Leu-Tyr 7-amido-4-methylcoumarin was shown by Woo et al. (21) to be cleaved by ClpP in the absence of ClpA. We synthesized the peptide Leu-Tyr-Arg-NH₂ (LYW-NH₂) and its succinylated derivative, replacing the carboxyl moiety of the fluorogenic substrate with tryptophan. LYW-NH₂ was an inhibitor of fluorogenic peptide cleavage by ClpP and of α-casein degradation by ClpAP (Table I). However, LYW-NH₂ and succinyl-LYW-NH₂ were not detectably degraded by ClpP. A synthetic pentapeptide variant of the tripeptide, Leu-Tyr-Leu-Tyr-Arg-NH₂ (LYLYW-NH₂), also inhibited both fluorogenic peptide and α-casein degradation (Table I). In contrast to LYW-NH₂, LLYYW-NH₂ was a good peptide substrate for ClpAP alone (Fig. 1, A and B). LLYYW-NH₂ was degraded into four different products by ClpP (Fig. 1A). The primary cleavage (>88%) occurred between Leu¹ and Tyr⁴, and slower cleavage occurred between Tyr⁴ and Phe⁵ (10-12%) (Fig. 1B). Each pair of products was obtained in approximately equimolar amounts, and no internal dipeptides or tripeptides were observed (Fig. 1A), indicating that each peptide molecule is cut only once and released. We also synthesized a series of modified peptides, XYLYW-NH₂, where X was Phe, Gly, Thr, Glu, or Lys. These pentapeptides were also cleaved at two alternative sites as shown for LLYYW-NH₂ in Fig. 1A. Alternative sites of cleavage could result because these peptide substrates can bind in more than one conformation at the active site of ClpP or because ClpP active sites can exist in more than one conformation. Evidence for heterogeneity in con-

**TABLE I**

<table>
<thead>
<tr>
<th>Peptide Substrate</th>
<th>Kᵢ (mM)</th>
<th>Peptidase Activity</th>
<th>Protease Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYW-NH₂</td>
<td>292</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>LLYYW-NH₂</td>
<td>40</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

**Figures**

1. **Fig. 1A** shows the cleavage of LYW-NH₂ and LLYYW-NH₂ by ClpP. The primary cleavage site between Leu¹ and Tyr⁴ is shown by the arrow. Secondary cleavages are indicated by dashed lines. 1. **Fig. 1B** shows the cleavage of LLYYW-NH₂ by ClpP. The primary cleavage site between Leu³ and Tyr⁴ is shown by the arrow. Secondary cleavages are indicated by dashed lines.

**Experimental Procedures**

See the original article for detailed experimental procedures and data analysis.
Clp Protease and Model Peptide Substrates

Fig. 1. Cleavage of hydrophobic pentapeptides by ClpP. LYLW-NH₂ and LYLW-CO₂H were synthesized as described under "Experimental Procedures." The purified peptides were succinylated (15), and the succinylated peptides were purified by reverse-phase chromatography. Peptides (50 μg) were incubated with 26 μg of ClpP under standard conditions in 250 μl for 3–6 h at 37 °C, and the products were analyzed by C₂₃, reverse-phase chromatography. A, absorbance trace at 290 nm at 6 h (broken line) and after 6 h (solid line) of incubation of LYLW-NH₂ with ClpP. The traces were normalized to enable visualization of the LYL peak at 18 min; the labeled product peaks accounted for 100% of the original peptide. Peptides were identified from their amino acid compositions, and their molar ratios were determined from their tyrosine and tryptophan contents using relative absorbance coefficients of 4 for tryptophan and 1 for tyrosine. About 15% deamination of YW-NH₂ occurred during incubation or during work-up of the reaction mixtures. B, cleavage sites in the modified pentapeptides after 3 h of digestion. Succ, succinyl.

Fig. 2. Competitive inhibition of peptide and protein degradation by hydrophobic pentapeptides. ATP-dependent protein degrading activity of ClpAP (A) was measured with 8 μg/ml α-casein, and peptidase activity of ClpP (B) was measured with a 1 mM concentration of the fluorogenic peptide N-succinyl-Leu-Tyr 7-amido-4-methylcoumarin (Succ-LY-AMC). Standard assay conditions (see "Experimental Procedures") were used with increasing concentrations of the pentapeptide X-Tyr-Leu-Tyr-Trp, where X was Phe (1), Leu (2), Gly (3), Thr (4), Glu (5), or Lys (6).

ClpAP Protease Cleaves Peptide Bonds between Amino Acids with Broad Range of Properties—Both glucagon and the oxidized insulin B chain are degraded by ClpAP in the presence of ATP. The kₘ for peptide bond cleavage in these polypeptides is comparable to that observed with α-casein and other proteins as substrates (see accompanying paper (20)). With the 30-amino acid hydrophobic insulin B chain, four of the five major cleavage sites occurred within or preceding hydrophobic regions (Fig. 3). However, cleavage occurred between Leu⁸ and cysteic acid 7 in essentially 100% of the molecules, indicating that negatively charged residues at the P1' position do not prevent cleavage. The sites cleaved in the more hydrophilic 29-amino acid peptide glucagon were more heterogeneous (Fig. 3). While two of the four major cuts were made before the hydrophobic residues Tyr¹⁹ and Trp²⁸, two other cuts occurred after the acidic residues Asp¹⁰ and Asp¹⁵, and one came after Ala¹³ within a hydrophilic region with charged residues flanking the amino acids at the cleavage site.

The above results indicate that Clp protease does not show strict specificity for the residues at the P1 or P1' position in substrates that are cleaved relatively slowly. The presence of a cluster of hydrophobic residues may facilitate entry of peptides or regions of proteins into the active site of ClpP, and the sites at which substrates are cleaved appear to depend on interactions of the protease with residues several positions removed from the cleavage site.

formation and substrate affinities of ClpP active sites is presented in the accompanying paper (22). The frequency of cutting at particular sites was affected by blocking the amino or carboxyl terminus of LYLW (Fig. 1B) and, to a lesser extent, by varying the amino-terminal amino acid (data not shown), suggesting that these modifications alter either the orientation of the peptides in the active sites or the choice of active sites where cleavage occurs. Alternate cleavage patterns were also seen with other peptide substrates, such as YGIPQINSR, which was cut by ClpP after Ile, Phe, or Gln (data not shown), and had been observed earlier for Lon protease in degrading λ N protein in vitro (26).
ClpAP-dependent Hydrolysis of ClpP Propeptide and Inhibition of α-casein Degradation—In searching for a more specific substrate for Clp protease, we used a known in vivo activity of ClpP to design a potential peptide substrate. In vivo, ClpP is made with a 14-amino acid propeptide sequence that is cleaved autocatalytically by ClpP to give the mature protease (8). A peptide corresponding to the first 19 amino acids of ClpP and a number of shortened forms truncated from the amino or carboxyl terminus were synthesized. These peptides, which contain the ClpP processing site, will be referred to as propeptides.

The full-length propeptide was rapidly cut by ClpAP, but not by ClpP alone (see below). Analysis of the degradation products indicated that the Met14-Ala15 bond, which is the processing site in vivo, was cut in 100% of the molecules (Fig. 4). There were two additional cuts in the amino-terminal region on either side of Tyr5.

The minimum sequence needed for recognition and cleavage was determined with the truncated propeptides. All propeptides that were degraded were cleaved exclusively at the Met14-Ala15 bond, which is the processing site, and was rapidly degraded only when ClpA, ClpP, and ClpAP in the presence of ATP (W), ATP-γ-S (.), or AMP-PNP (A). Degradation was measured by the decrease in the FAPHMALVPV peak in samples separated by reverse-phase chromatography.

ClpAP cleaves FAPMVPV in presence of nonhydrolyzable analogs of ATP. FAPMVPV was incubated with ClpP (P), with ClpP and ClpA in the absence of nucleotide (V), and with ClpA in the presence of ATP (□), ATP-γ-S (○), or AMP-PNP (△). Degradation was measured by the decrease in the FAPMVPV peak in samples separated by reverse-phase chromatography.

Substrate and Inhibitor Constants Suggest Two Sites for ClpP Propeptide on ClpAP Protease—The propeptide and its shortened derivatives were effective competitors in the α-casein degradation assay (Fig. 4). The full-length propeptide as well as DNFPAPMVPV and FAPMVPV had similar inhibitor constants, but amino-terminal truncation beyond Phe10 or truncation of the 3 carboxyl-terminal amino acids caused a significant, although only 3–5-fold, drop in affinity. Propeptides either beginning with Met14 or ending with Ala15, with only a single amino acid on either side of the scissile bond, did not inhibit α-casein degradation even at millimolar concentrations. FAPH0-MVPV was as effective as FAPMVPV in inhibiting α-casein degradation (Fig. 4 and Table I). Surprisingly, none of the propeptides inhibited fluorogenic peptide degradation by ClpP in the absence or presence of ClpA. These data suggest that not all active sites of ClpP are available simultaneously to different substrates (see accompanying paper (20)).

Table I shows the values for the full-length propeptide and FAPMVPV compared with the values for these peptides as...
inhibitors of α-casein degradation. The $K_a$ values were in the millimolar range (5–8 mM), while the $K_v$ values were calculated to be in the micromolar range (15–25 μM). This discrepancy between the $K_a$ and $K_v$ values for the peptides could reflect the presence of a second binding site for peptides on ClpAP, separate from the catalytic site, at which competition with α-casein occurs. Evidence for such a site on the ATPase subunit, ClpA, is presented below. Alternatively, a kinetic mechanism in which equilibrium binding of substrates is slow compared with the catalytic rate could also give rise to a difference in $K_a$ and $K_v$ values (see “Discussion”).

Effects of Substituting Amino Acids at P1, P2, and P3 Positions on Propeptide Degradation by ClpAP—Using FAPHALVPV as a model, peptides were synthesized in which Met, His, and Pro, which occupy the P1, P2, and P3 positions relative to the cleavage site, respectively, were replaced by different amino acids. Peptides with Trp, Ile, Lys, Ser, or Glu in place of His or Ile or Gly in place of Pro were degraded at the Met–Ala bond (Table III); degradation required ClpA, ClpP, and nucleotide. Degradation rates for the peptides in which His was replaced followed the order Ile > Trp > Lys > Ser > Glu, with only the Glu-substituted peptide showing a marked (60%) decrease (Table III). The substituted propeptides also inhibited α-casein degradation in the following order: Trp = Ile = Lys ($K_{in} = 47 μM$) > Ser = Glu ($K_{in} = 63 μM$). The bend in the peptide backbone of FAPHALVPV caused by Pro is not important for specificity or efficiency of cleavage since replacement of Pro with Ile increased the rate of cleavage of the Met–Ala bond.

Replacement of Met, however, had a drastic effect on degradation of the peptide. Of seven different substitutions, only peptides with Leu or Trp in place of Met were degraded to a measurable extent (Table IV). Cleavage of the substituted peptides came at the same relative position as on the propeptide, between the Leu–Ala or Trp–Ala bond (data not shown), indicating that the proteolytic active site can accommodate an extended hydrophobic side chain in the P1 position. The stringent preferences for Trp but not Tyr and for Leu but not Ile at the P1 position was surprising especially since cleavage after Tyr occurs with the fluorogenic peptide and the pentapetides. These results may reflect the role played by other residues in the peptide in positioning the scissile bond for cleavage or might indicate a difference in the properties of the ClpP active site when it is alone or complexed with ClpA. All the peptides inhibited α-casein degradation with only slight differences in affinity for binding at the allosteric site (Table IV).

Degradation of Peptides with Different Amino Acids Substituted for the Methionine at the P1 Position was Measured after 10 min in the Presence of 2 μg of ClpA, 0.2 μg of ClpP, and 1 mM substrate. The amount of degradation observed was normalized to that observed with the methionine-containing propeptide. Inhibition constants were determined with α-casein as the substrate.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative rate of degradation by Clp</th>
<th>$K_v$</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAPHALVPV</td>
<td>1.00</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>FAPHALVPV</td>
<td>0.12</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>FAPHALVPV</td>
<td>0.08</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>FAPHALVPV</td>
<td>0.00</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>FAPHALVPV</td>
<td>0.00</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>FAPHALVPV</td>
<td>0.00</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>FAPHALVPV</td>
<td>0.00</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

Degradation of Tandem ClpP Propeptide (FAPHALVPVFAPHALVPV) Is ClpAP-dependent—As a further test of the specificity of the cleavage at the Met–Ala bond, we synthesized a peptide composed of FAPHALVPV repeated in tandem and tested it as a substrate. We also made tandem peptides in which either 1 of the 1-Met residues was replaced by d-Met. FAPHALVPVFAPHALVPV was cleaved specifically between Met and Ala at both sites, although the rate of cleavage...
Effects of proteins and peptides on ClpA ATPase activity

<table>
<thead>
<tr>
<th>Protein/peptide</th>
<th>ATPase activity</th>
<th>$K_v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>%</td>
</tr>
<tr>
<td>$\alpha$-Casein</td>
<td>145</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>FAPHMALVPV</td>
<td>144</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>FAPHMALVPVFAPHMALVPV</td>
<td>145</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

The effects of proteins and peptides on ClpA ATPase activity have been studied. ATPase activity of ClpA was measured by the release of $^{32}$P phosphorlate from ATP, ATPase activity with ClpA alone was ~60 nmol/h/μg. Casein and peptides were added at a final concentration of 500 μg/ml.

was slower (one-tenth) than that observed with FAPHMALVPV (Table II). The three peptide products from the tandem peptide accumulated with identical kinetics, indicating that both sites are cleaved in each peptide. With the tandem peptides containing p-Met at one or the other position, cleavage occurred only between the p-Met-Ala bond and at rates comparable to those seen with the tandem peptide (Table II). Interestingly, the tandem peptides had much lower $K_v$ values (100-300 μm) than observed for the propeptides, while the tandem propeptide concentration required for half-maximal inhibition of $\alpha$-casein degradation (22-36 μm) was comparable to that for the propeptide (27 μm) (Table II). The lower $K_v$ values seen for substrates that are cleaved at lower $k_{cat}$ values lend support to a kinetic model in which the $k_{cat}$ is comparable to or greater than the off-rate in the substrate binding reaction (see "Discussion" for details).

**Effects of ClpP Propeptide on ClpA ATPase Activity**—The kinetic constants for the propeptide suggested that there could be more than one site for binding of peptides on ClpAP. Earlier studies had indicated that proteins might interact directly with ClpA (9, 27), and we therefore tested the effects of FAPHMALVPV and some of its modified forms on the basal ATPase activity of ClpA in the absence of ClpP. All forms of the propeptide activated ClpA ATPase activity by ~45%, similar to the activation seen with $\alpha$-casein alone (Table V). Activation of ClpA ATPase occurs at peptide concentrations comparable to the $K_v$ for inhibition of $\alpha$-casein degradation, indicating that the allosteric site for peptide and protein (casein) binding on ClpAP lies within the ClpA subunit.

### DISCUSSION

Although peptide bond turnover rates have been reported for non-ATP-dependent cleavage reactions by the eukaryotic 20 S proteasome, such as degradation of the insulin B chain (28) and cleavage of various small peptides (29, 30), and for the ATP-dependent cleavage by Lon protease of E. coli (26, 31), this is the first report of quantitative data for a multiple-component ATP-dependent protease. The turnover number for propeptide cleavage (800 min$^{-1}$/substrum or 9600 min$^{-1}$/dodecamer of ClpP) is 10-100 times faster than rates reported for the ATP-dependent Lon protease (26, 31) and at least 10 times faster than rates reported for the 20 S proteosome (28). The ClpAP complex cleaves the Met-Ala bond in a specific peptide 20-40 times faster than specific sites in protein and peptide substrates and ~100 times faster than cleavage of small peptides by ClpP alone. The very large rate enhancement for propeptide cleavage depends on the interaction of the peptide with ClpA and on the enhanced active site of ClpP provided by the complex of ClpA and ClpP.

The proteolytic active sites of Clp protease reside on ClpP subunits. Very short peptides can be cleaved by ClpP alone (this paper and Refs. 18 and 21), and the insulin B chain (30 amino acids) is degraded by ClpP at ~2% the rate seen with ClpAP (see accompanying paper (20)). ClpA is, however, absolutely required to activated ClpP for degradation of larger proteins such as casein and globin (9, 12, 32). Our studies indicate that whether ClpA is required for peptide cleavage depends not only on the size of the peptide substrate, but also on its composition. As reported here, cleavage of truncated propeptides as short as 7 amino acids requires both ClpA and ClpP, and the ClpAP-dependent degradation of propeptides occurs at 10-200 times the rate of fluorogenic peptide or pentapeptide degradation by ClpP. Thus, the composition of even short peptides affects their ability to get into and be cleaved at the active site of ClpP.

While a role for ClpA in activating degradation of proteins may be understood in terms of a chaperone or unfolding activity, activation of peptide degradation by ClpA, which requires nucleotide binding but not ATP hydrolysis, suggests that other mechanisms may be involved as well. The nucleotide-promoted interaction of ClpA with ClpP increases catalytic efficiency at the ClpP active site. This effect could result from a conformational change that "opens" or optimizes the active site of ClpP, as has been suggested for the ATP-dependent Lon protease, for which nucleotide binding greatly activates cleavage of peptides (6, 31). ClpA could also provide a channel by which specific peptides have preferential access to the active site. These models are discussed further in the accompanying paper (20). Recently, a high molecular weight protein complex, PA700, was shown to bind to the 20 S proteasome in an ATP-dependent manner and to activate peptidase activity of the proteasome without additional ATP (33). It is possible that ClpP and the proteasome may both be allosterically activated by interaction with their respective regulatory components.

As is the case with Lon protease (28), the subsites in the proteolytic active site on ClpP can accommodate or tolerate a number of different side chains. This lack of strict amino acid specificity is apparent from the different amino acids surrounding the cut sites in the insulin B chain and glucagon. Cleavage of the pentapeptides at two alternative sites suggests that the same peptide can be oriented in the active site with different peptide bonds positioned to be cleaved. It is also possible that subunit interactions in the dodecamer of ClpP affect the conformations of active sites on different subunits (see accompanying paper (20)). The orientation of peptides within the active site is affected by interactions between residues on either side of the scissile bond. In the case of Clp, those on the carboxy-terminal side may have particular influence, as shown by the differences in cleavage sites in the pentapeptide amides compared with the acids and by the altered cleavage of the propeptide when the carboxy-terminal amino acids are replaced.

Mutational studies suggest that the same active site of ClpP is used for autoprocessing and protein degradation (34). We have shown that a synthetic peptide that includes the ClpP propeptide sequence plus the next 5 amino acids is a unique substrate for ClpAP protease. First, cleavage occurs at the precise Met-Ala bond cleaved in vivo, and second, cleavage is much faster than any other substrates. The most rapidly degraded peptide, FAPHMALVPV, was turned over at 300 min$^{-1}$. This rate is very much higher than those for Clp protease-degrading substrates such as casein and the insulin B chain (see accompanying paper (20)) and is much higher than the turnover rates for Lon protease with casein (~2 min$^{-1}$) (31) or with a physiological substrate, λ N protein (~60 min$^{-1}$) (26).

The full-length and truncated propeptides as well as the tandem propeptide FAPHMALVPVFAPHMALVPV were cleaved only between the Met-Ala peptide bond. The explanation for the remarkable specificity of this cleavage is still elusive. Leu and Trp could replace Met at the P1 position, suggesting that the subsite at which the P1 residue binds has an extended hydrophobic pocket. Even these conservative substi-
tutions, however, produced a >85% decrease in degradation rate. Peptides with hydrophilic or charged residues at the P1 position apparently cannot be positioned for cleavage in the active site. The relatively minor effects of altering His at the P2 position or Pro at the P3 position or removing the Phe at P4 and Ala at P5 suggest that these positions do not provide essential positive interactions for binding or positioning of the propeptide. This strict orientation of the scissile bond in the active site of ClpP stands in contrast to the rather promiscuous cleavage site selection in the pentapeptide substrates or insulin B chain. It is interesting that, although an Ala-Leu bond is readily cleaved in the insulin B chain, this bond is not cut in any form of the propeptide, including those with Pro, His, or Met substitutions. A change in absolute specificity of cleavage was seen only with position apparently cannot be positioned for cleavage in the ac-

N-acetylated proteins are recognized by components of the deg-

ative machinery separate from those that recognize free peptide were nonproductive and required some conformational change in the enzyme to orient the substrate to, for example, enter an access channel properly. Binding studies to determine k1 and k-1 are underway.

It is likely that the inhibition of ClpAP-dependent α-casein degradation by the propeptides is due to competition between the ClpP propeptide and α-casein for binding to a site on ClpA. It was reported earlier (9, 12) that both proteins and peptides affect the ATPase activity of ClpA in the absence of ClpP, indicating that ClpA has a binding site for protein substrates. Our results show that propeptide derivatives and α-casein stimulate ClpA ATPase activity to a similar extent, and preliminary data suggest that they bind to either identical or overlapping sites on ClpA (data not shown). Activation of ClpA ATPase activity occurs at concentrations comparable to the Kd for inhibition of α-casein degradation and indicates that the allostERIC site for peptide and protein (α-casein) binding on ClpAP protease lies within the ClpA subunit. The direct interaction of peptide and protein substrates with ClpA is consistent with models that have been proposed in which ClpA (and possibly other Clp ATPases) serve as a molecular chaperone to present substrates to ClpP or other proteolytic components for degradation (1, 17, 19, 32, 36).

The different interactions of these model peptide substrates with ClpP and ClpAP protease clearly demonstrate the complexity of studying this proteolytic system; no simple rules for recognition of peptides and for selection of peptide bonds to be cleaved can be made. ClpAP is able to cleave the Met-Ala bond of the propeptide extremely rapidly, but can cleave a variety of other peptide bonds at slower rates. The interaction of substrates at the allostERIC site on ClpA (and more weakly but very specifically at the active site on ClpP) appears to be characteristic of the best substrates for ClpAP protease.

Acknowledgment—We are grateful to Susan Gottesman for helpful discussions and comments on the manuscript.

REFERENCES