Purification and Characterization of Protease Ci, a Cytoplasmic Metalloendoprotease in Escherichia coli*

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Protease Ci, a cytoplasmic metalloprotease in Escherichia coli, has been purified to apparent homogeneity by conventional chromatographic procedures using 125I-labeled oxidized insulin B-chain as a substrate. The purified enzyme behaves as a 54-kDa protein under both denaturing and non-denaturing conditions, suggesting that it consists of a single polypeptide chain. It is inhibited by metal-chelating agents, including o-phenanthroline and NaCN, but not by inhibitors of serine proteases or thiol-blocking agents. Furthermore, protease Ci was found to contain 1.1 mol of zinc per mol of the enzyme upon analysis by HR ICP mass spectroscopy. Thus, protease Ci must be a zinc metalloprotease. Among the polypeptides tested as substrates, oxidized insulin B-chain and glucagon are most rapidly hydrolyzed. Intact insulin is a much poorer substrate than oxidized insulin B-chain, even though the affinity of the enzyme to intact insulin is approximately 100-fold greater than to the B-chain. Since unlabeled oxidized insulin A-chain is capable of inhibiting the hydrolysis of 125I-labeled insulin B-chain, it also appears to be a substrate. Protease Ci also degrades lysozyme and lactalbumin, although to a much lesser extent than oxidized insulin B-chain. However, it shows little or no activity against proteins larger than 15 kDa (e.g. ovalbumin and denatured bovine serum albumin). Hydrolysis of oxidized insulin B-chain followed by amino acid composition analyses of the cleavage products reveals that as many as 10 of its 29 peptide bonds are hydrolyzed by protease Ci. This ability to hydrolyze relatively small polypeptides suggests that protease Ci may catalyze the later steps in the pathway for intracellular protein breakdown.

Soluble extracts of Escherichia coli contain at least nine distinct endoproteases that appear to be distinct from each other (1, 2). Proteases Do (DegP), Re (Tsp), Mi, Fa, So, La (Lon), and Ti (Clp) are serine proteases that hydrolyze relatively large proteins, such as casein and globin. Of these, proteases La and Ti require ATP and Mg2+ for activity and appear to catalyze the rate-limiting steps in the hydrolysis of abnormal proteins and of certain normal proteins (3, 4). Two other enzymes, proteases Ci and Pi, are metalloproteases that degrade smaller polypeptides, such as insulin and the N-terminal fragment of β-galactosidase with a minimal length of 50 amino acids (called “auto-α”) that were used for complementation assay of β-galactosidase upon mixing with appropriate acceptor proteins (2, 5). Proteases M1 and Pi are periplasmic enzymes, while all others are localized to the cytoplasm and therefore may play a role in the degradation of intracellular proteins (6).

The periplasmic insulin-degrading protease Pi, also called protease III (5) and piritilysin (7), has been extensively characterized, although its physiological function is still unknown. Interestingly, protease Pi shares structural and functional homologies with the cytosolic insulin-degrading enzymes present in most mammalian cells (8). Overall amino acid sequence similarity between these enzymes is about 50% when conserved amino acid changes are included as matches. Furthermore, both the proteases contain three highly conserved domains, of which the first domain is characterized by the presence of a metal-binding site (9). Therefore, it has been suggested that the three regions of homology may play an important role in the active site and catalytic mechanism of the proteases (8).

In addition to sharing sequence similarity, both protease Pi and human insulin-degrading enzyme have a molecular mass of about 110 kDa and require a divalent metal ion for activity (5, 8, 9). Both proteases have an isoelectric point of 5.3 and are maximally active in the pH range of 6.5–8.5. Both are sensitive to inhibition by bacitracin. However, they differ in sensitivity to sulfhydryl blocking agents, such as N-ethylmaleimide and iodoacetamide. While the human insulin-degrading enzyme is highly sensitive to these reagents, protease Pi is not. An insulin-degrading enzyme similar in its properties to the human enzyme and protease Pi has also been isolated from Drosophila melanogaster (10).

E. coli in its cytoplasm contains an additional enzyme, protease Ci, that can degrade insulin (1, 2). Aside from its subcellular location, protease Ci shows remarkable similarities to protease Pi. Like Pi, it appears to be 110–130 kDa, to have a pH optimum at 7.5, and to be sensitive to inhibition by metal-chelating agents and by the antibiotic bacitracin. However, all these properties were determined only on the partially purified protease Ci, whose physiological role is totally unknown. In the present studies, therefore, we purified protease Ci completely to carry out a detailed characterization of its biochemical and physicochemical properties, for determination of its cleavage specificity, and ultimately for elucidation of its physiological function.

EXPERIMENTAL PROCEDURES

Materials—E. coli 3302 strain (HfrH ptr-3 lacZ77) (11) was grown in Luria broth, harvested at late log phase, and kept at −70 °C until use. Protein substrates including insulin and oxidized insulin B-chain were radioiodinated using Iodo-Beads as described (12). Denatured
bovine serum albumin (BSA) \(^1\) was prepared by reduction of disulfide bonds in the presence of 6 m guanidine HCl and alkylation with iodoacetamide (13). Iodo-Beads were obtained from Pierce; HiLoad Q, heparin-Sepharose, and phenyl-Sepharose were from Pharmacia Biotech Inc.; butyl-Toyopearl and TSK gel ODS-80TM column were from TOSOH Corp. (Japan). All others were purchased from Sigma.

Assays—Proteolysis was assayed as described (14). The reaction mixture (final volume, 0.1 ml) contained 50 mM Tris-HCl (pH 8), 5 mM MgCl\(_2\) and proper amounts of \(^{125}\)I-labeled protein substrates and the protease preparations. Incubations were performed at 37 °C for 30–60 min. The amount of radioactive materials soluble in 10% (v/v) trichloroacetic acid was then determined using a γ counter. Proteins were assayed as described by Bradford (15) using BSA as a standard. Isoelectric focusing of proteins on 5% (w/v) polyacrylamide gels was carried out as described (16).

Preparation of Crude Extract—The frozen E. coli cells (140 g) were thawed and suspended in 50 ml of buffer A (20 mM Tris-HCl (pH 7.8) and 5 mM MgCl\(_2\) containing 100 mM NaCl). The cells were disrupted with a French press at 14,000 psi and centrifuged at 100,000 × g for 3 h. The supernatant was dialyzed against the same buffer and referred to as crude extract.

Preparation of Antibody—The purified protease Ci preparation obtained from the final purification step (see below) was subjected to electrophoresis on 10% polyacrylamide slab gels containing SDS and 2-mercaptoethanol (17). After briefly staining the gels with Coomassie Blue R-250, the protein band of the 54-kDa protease Ci was cut off and crushed. The sample was then subcutaneously injected into albino rabbits for the production of anti-protease Ci antiserum. IgGs were isolated from the antiserum by sodium sulfate fractionation (18). Immunoblot analysis was performed using the antiserum or the anti-IgG as described (19).

Determination of Cleavage Specificity—Oxidized insulin B-chain was digested with the purified protease Ci for 0.5–15 h at 37 °C. The cleavage products were subjected to reversed-phase HPLC using a C\(_{18}\) column (TSK gel ODS-80TM, 4.6 × 150 mm). The peptides bound to the column were eluted with a linear gradient of 1–35% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Elution of the peptides was monitored by their absorbance at 206 nm. Each of the peptide peaks was pooled, concentrated by evaporation, and again subjected to the reversed-phase HPLC. Homogeneous peptides were isolated, dried, and hydrolyzed in 6 N HCl and 0.1% (v/v) phenol at 140 °C for 4 h under vacuum. The amino acid compositions of the peptides were then determined using an HPLC system (TOSOH Corp.) equipped with a fluorescent detector (model FS8010) and a system controller (data processor, model SC8020).

Determination of Metal Ion—The purified protease Ci was extensively dialyzed against Milli-Q water with resistance of 18 megohm. The Milli-Q water from the last dialysis was used as the reference. All the glasswares used in this study were boiled in 5% (v/v) HCl for 3 h to remove adventitious metal ions. Metal contents of the enzyme were then determined using an HR ICP mass spectrometer (model VG Plasma Trace).

\(^1\) The abbreviations used are: BSA, bovine serum albumin; HPLC, high performance liquid chromatography.

RESULTS

Purification—The crude extract (17.7 g of protein) was obtained from 140 g of E. coli 3302 strain that lacks protease Pi, degrading insulin and existing in the periplasmic space (2, 5). The extract was loaded onto a DEAE-Sepharose column (5 × 20 cm) equilibrated with buffer A containing 100 mM NaCl. After washing the column, proteases were eluted with a linear gradient of 100–250 mM NaCl and assayed for proteolysis. As shown in Fig. 1, a single peak of proteolytic activity appeared at about 170 mM NaCl, whether \(^{125}\)I-labeled insulin or oxidized insulin B-chain was used as the substrate. However, oxidized insulin B-chain was hydrolyzed at a rate at least 50-fold faster than intact insulin (also see below). Therefore, we used oxidized insulin B-chain as the substrate for further purification and characterization of protease Ci, although this enzyme had initially been identified using intact insulin as its substrate (2, 20).

The fractions containing high activity were pooled and applied to a HiLoad Q column (2.6 × 10 cm) equilibrated with buffer A containing 150 mM NaCl. Proteins bound to the column were eluted with a linear gradient of 150–300 mM NaCl. The active fractions were pooled, dialyzed against buffer A, and loaded onto a heparin-Sepharose column (1.5 × 8 cm) equilibrated with the same buffer. Proteins that did not bind to the column were collected, and a saturated (NH\(_4\))\(_2\)SO\(_4\) solution (pH 7.8) was added to a final concentration of 1 M. The sample was then loaded onto a phenyl-Sepharose column (1 × 6 cm) equilibrated with buffer A containing 1 M (NH\(_4\))\(_2\)SO\(_4\). Proteins bound to the column were eluted by decreasing linearly the salt concentration to 0.01 M. The peak of oxidized insulin B-chain-degrading activity was eluted at about 0.17 M (NH\(_4\))\(_2\)SO\(_4\) (Fig. 2A). The fractions containing high activity were pooled, adjusted to 1.2 M (NH\(_4\))\(_2\)SO\(_4\), and loaded onto a butyl-Toyopearl column (1 × 4 cm) equilibrated with buffer A containing 1.2 M (NH\(_4\))\(_2\)SO\(_4\). Proteins were eluted by decreasing the salt concentration to 0.1 M. Active fractions were pooled, adjusted to 1.5 M (NH\(_4\))\(_2\)SO\(_4\), and again loaded onto the same butyl-Toyopearl column but equilibrated with 1.5 M (NH\(_4\))\(_2\)SO\(_4\). Proteins were eluted as above but by decreasing the salt concentration to 0.9 M. The fractions containing high activity (Fig. 2B) were pooled, added with glycerol to a final concentration of 20% (v/v), and kept at −70 °C for further use. Summary of the purification of protease Ci is shown in Table I.

Physicochemical Properties—The subunit size of protease Ci was estimated to be 54 kDa upon analysis by polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol (Fig. 3A, lane b). Only a single band could be seen in the
Protease Ci, a Cytoplasmic Metalloprotease in E. coli

Fig. 2. Elution profiles of the protease Ci activity from phenyl-Sepharose (A) and the second butyl-Toyopearl columns (B). Proteins obtained from heparin-Sepharose column (17.2 mg) were chromatographed on the phenyl-Sepharose column. Fractions of 1 ml were collected at a flow rate of 10 ml/h. Proteins obtained from the DEAE-Sepharose column (see Fig. 1) were also subjected to gel filtration using the Superose-12 column. As shown in Table II, o-phenanthroline at 1 mM inhibited the enzyme activity by about 50%. NaCN, which also is known to inhibit metalloproteases (21), reduced the activity by about 80% at 1 mM. In addition, the antibiotic bacitracin at 0.7 mM inhibited the enzyme by about 90%. However, protease Ci was not sensitive to inhibitors of serine proteases, such as diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, or of sulfhydryl proteases, including N-ethylmaleimide and iodoacetamide. It is also not sensitive to various antibiotic peptide aldehyde inhibitors tested, such as leupeptin, antipain, chymostatin, and bestatin, nor to the epoxide, E-64. Thus, protease Ci appears to be a metalloprotease. Nearly identical results were obtained when intact insulin was used as the substrate (data not shown).

To clarify further the requirement for metal ions, protease Ci was preincubated with 0.2 mM NaCN for 20 min, which blocked the initial enzyme activity by about 65%. Various metal ions were then added to determine whether any of them can reactivate the enzyme. As shown in Table III, protease Ci could be reactivated by Co2+, Mn2+, and Cu2+ in the order of their effectiveness but not by Ca2+, Mg2+, and Zn2+ at the concentrations tested. Without the preincubation, however, Zn2+, Co2+, and Cu2+ strongly inhibited the activity of protease Ci while Mg2+, Ca2+, and Mn2+ were stimulatory (data not shown). Thus, it appeared as if protease Ci uses Co2+ as its metal cofactor.

To determine whether protease Ci indeed contains Co2+, HR-ICP mass spectral analyses of two different preparations of the purified enzyme were performed. The enzyme contained 1.51 and 1.26 μg of Zn2+ per mg of protein. Assuming a size of 54 kDa, this is equivalent to a mean of 1.1 mol of Zn2+ per mol of protease Ci. Although the NaCN-treated enzyme could be reactivated most effectively by Co2+ and not by Zn2+, it is a well known fact that metalloproteases that use Zn2+ as the natural metal cofactor can be inhibited when incubated with excess Zn2+. In addition, in some cases (e.g. metallocarboxypeptidases), Co2+ activates the enzyme severalfold over the activity seen with the native Zn2+-containing enzyme (22-24). Thus, protease Ci is likely to be a Zn2+ metalloprotease.

Hydrolysis of Protein Substrates—To determine the time course for hydrolysis of 125I-labeled insulin and oxidized insulin B-chain, the purified protease Ci was incubated with these substrates for varying periods. As shown in Fig. 5, hydrolysis of both polypeptides increased linearly for the duration of incubation. In addition, this result again shows that oxidized insu-
lin B-chain is much more rapidly degraded than intact insulin. From these data, the specific activity of protease Ci against intact insulin was estimated to be 2.5 nmol/min/mg, which is comparable to that of Drosophila insulin-degrading enzyme (3.3 nmol/min/mg) (10).

We then examined whether protease Ci can also hydrolyze oxidized insulin A-chain. The activity of the purified enzyme against 125I-labeled B-chain was measured in the presence of increasing amounts of the unlabeled A-chain. As shown in Fig. 6, the insulin A-chain (like unlabeled intact insulin and oxidized B-chain) could competitively reduce breakdown of the radiolabeled B-chain. These results suggest that protease Ci is also capable of hydrolyzing oxidized insulin A-chain. In addition, unlabeled intact insulin molecules were able to inhibit the hydrolysis of 125I-labeled B-chain, even more effectively than any of A- or B-chain. From these data, the $K_i$ values of the competing, unlabeled intact insulin and oxidized insulin B-chain molecules were estimated to be 0.13 and 12.4 $\mu M$, respectively. These results suggest that the affinity of protease Ci for intact insulin is about 100-fold higher than that of oxidized insulin B-chain, despite the fact that the latter polypeptide is much more rapidly hydrolyzed by the enzyme than intact insulin.

A variety of 125I-labeled proteins of different sizes was also tested for susceptibility to the purified protease Ci. Glucagon was also rapidly degraded by the enzyme but approximately 20% as fast as oxidized insulin B-chain (Table IV). Protease Ci was also capable of degrading polypeptides with sizes smaller

### TABLE I

| Step              | Protein | Total activity (unit) | Specific activity (unit/mg) | Yield % | Purification (-fold) |
|-------------------|---------|----------------------|-----------------------------|---------|----------------------|
| Crude extract     | 17732   | 4080                 | 0.23                        | 100     | 1                    |
| DEAE-Sepharose    | 410     | 2448                 | 6                           | 60      | 26                   |
| HiLoad Q          | 25.2    | 1217                 | 48                          | 30      | 209                  |
| Heparin-Sepharose | 17.2    | 970                  | 56                          | 24      | 243                  |
| Phenyl-Sepharose  | 2.5     | 316                  | 126                         | 7.7     | 548                  |
| 1st butyl-Toyopearl | 0.66   | 295                  | 447                         | 7.2     | 1943                 |
| 2nd butyl-Toyopearl | 0.09   | 62                   | 689                         | 1.5     | 2996                 |

### FIG. 3

**Gel electrophoretic (A) and immunoblot analyses (B) of the cell lysate and the purified protease Ci.** E. coli 3302 strain was grown to mid-log phase, harvested, and immediately boiled in 2% SDS. An aliquot of the cell lysate (lane a) and 7 $\mu$g of the purified protease Ci (lane b) were electrophoresed in duplicates on 10% polyacrylamide slab gels containing SDS and 2-mercaptoethanol. One of the gels was stained with Coomassie Blue R-250. The other gel was transferred onto a nitrocellulose paper and subjected to immunoblot analysis using the anti-protease Ci antiserum. The arrows indicate the position where protease Ci migrated in the gels.

### FIG. 4

**Gel filtration of the protease Ci preparations on a Superose-12 column.** The purified protease Ci (●) and the enzyme preparation obtained from the DEAE-Sepharose column of Fig. 1 (E) were chromatographed on a Superose-12 column (1 × 30 cm) equilibrated with buffer A containing 100 mM NaCl. Fractions of 0.5 ml were collected at a flow rate of 20 ml/h, and an aliquot was assayed for hydrolysis of 125I-labeled insulin B-chain. Size markers used were as follows: a, $\beta$-amylase (200 kDa); b, alcohol dehydrogenase (150 kDa); c, BSA (66 kDa); d, carbonic anhydrase (29 kDa).

### TABLE II

| Inhibitors             | Concentration | Relative activity (%) |
|------------------------|---------------|-----------------------|
| None                   | 100           |
| o-Phenanthroline       | 1 mM          | 43                    |
| NaCN                   | 1 mM          | 18                    |
| Phenylmethylsulfonyl fluoride | 1 mM | 100                  |
| Diisopropylfluorophosphate | 1 mM | 105                  |
| N-Ethylmaleimide       | 1 mM          | 99                    |
| Iodoacetamide          | 1 mM          | 98                    |
| Bacitracin              | 0.7 mM        | 13                    |
| E-64                   | 5 $\mu$g/ml   | 89                    |
| Leupeptin              | 5 $\mu$g/ml   | 103                   |
| Antipain               | 5 $\mu$g/ml   | 108                   |
| Bestatin               | 5 $\mu$g/ml   | 106                   |

Protease Ci (0.1 $\mu$g) was incubated for 20 min at 37 °C with the inhibitors prior to the addition of 5 $\mu$g of 125I-labeled insulin B-chain. Protodexylosis was then assayed by further incubation of the mixtures for 30 min as described under “Experimental Procedures.” The enzyme without added inhibitor converted 1.9 $\mu$g of the B-chain into trichloroacetic acid-soluble products. This activity was expressed as 100%, and the others were as their relative values.
than 15 kDa, such as lactalbumin and lysozyme, although to a much lesser extent than oxidized insulin B-chain. However, it showed little or no activity against any larger proteins tested, such as BSA, denatured BSA, ovalbumin, carbonic anhydrase, and globin. In addition, protease Ci cleaved angiotensin I and the synthetic peptide that is used as a substrate of a tyrosine-protein kinase (RRLIEDAEYAARG) upon analysis of their incubation mixture on HPLC. However, it failed to cleave Leu-enkephalin and bradykinin. Therefore, it is tempting to speculate that protease Ci may preferentially hydrolyze the peptides with amino acids ranging from 10 to 150.

### Table III

Inhibition of the activity of protease Ci by NaCN and its reversal by various metal ions

| Addition       | Concentration | Insulin B-chain hydrolysis % |
|----------------|---------------|------------------------------|
| None           | 0.2           | 38.1                         |
| NaCN alone     | 0.2           | 13.0                         |
| With Mg<sup>2+</sup> | 1.0       | 128                          |
| With Ca<sup>2+</sup> | 0.2       | 18.5                         |
| With Mn<sup>2+</sup> | 1.0       | 19.1                         |
| With Co<sup>2+</sup> | 0.2       | 38.6                         |
| With Cu<sup>2+</sup> | 0.4       | 42.0                         |
| With Zn<sup>2+</sup> | 0.2       | 28.1                         |
| With BSA       | 0.4           | 43.5                         |
| With Denatured BSA | 0.4    | 31.9                         |

![Fig. 5. Time-dependent hydrolysis of insulin and oxidized insulin B-chain by the purified protease Ci.](image)

**TABLE IV**

Hydrolysis of various proteins by protease Ci

| Substrate                  | Specific activity (nmol hydrolyzed/mg/min) |
|----------------------------|------------------------------------------|
| Oxidized insulin B-chain   | 187                                      |
| Glucagon                   | 39                                       |
| Insulin                    | 2.5                                      |
| Lactalbumin                | 0.47                                     |
| Lysozyme                   | 0.21                                     |
| Casein                     | 0.03                                     |
| Globin                     | 0                                        |
| Carbonic anhydrase         | 0                                        |
| Ovalbumin                  | 0                                        |
| BSA                        | 0                                        |
| Denatured BSA              | 0                                        |

![Fig. 6. Effects of unlabeled insulin chains and BSA on the hydrolysis of 125I-labeled insulin B-chain by protease Ci.](image)

Protease Ci, a Cytoplasmic Metalloprotease in E. coli

Significantly, thus, it appears likely that the peptides in the former peaks behave as intermediates in degradation by protease Ci. When the individual peaks were red chromatographed on the same column but using a shallow gradient of acetonitrile, each of the peaks 4, 8, and 12 was further separated into two distinct peaks (data not shown).

The cleavage sites on oxidized insulin B-chain were then determined by analysis of the amino acid composition of each of the purified peptides. Fig. 8 summarizes the amino acid sequences of the peptides deduced from their amino acid compositions. In accord with the results from the reversed phase chromatography, a number of peptides appeared to behave as intermediates in degradation, such as peptide 8b for peptides 1 and 4a, 12b for 2 and 11, and 14 for 2 and 13. Peptide 5 may also be an intermediate in the generation of peptide 1 and LCGSH. However, the latter pentapeptide was not found among the 14 peaks and may correspond to any of the minor peaks whose amino acid composition was not determined. These results strongly suggest that protease Ci preferentially degrades the amino side of hydrophobic amino acids (i.e. the peptide bonds of His<sup>5</sup>-Leu<sup>6</sup>, His<sup>10</sup>-Leu<sup>11</sup>, Leu<sup>11</sup>-Val<sup>12</sup>, Glu<sup>13</sup>,...
Protease Ci, a Cytoplasmic Metalloprotease in E. coli

In the present studies, a cytoplasmic metalloprotease in E. coli, protease Ci, was purified to apparent homogeneity using $^{125}$I-labeled oxidized insulin B-chain as a substrate. Since E. coli contains an additional insulin B-chain-degrading enzyme, protease Pi, in the periplasm (2, 6), we used E. coli 3302 strain, which lacks the periplasmic enzyme. From 140 g of the frozen cells, we obtained 90 $\mu$g of purified protease Ci with a final yield of about 1.5%. Thus, protease Ci accounts for at least 0.03% of the soluble protein of E. coli.

Protease Ci was found to have much higher affinity for intact insulin than for oxidized insulin B-chain, despite the fact that the latter polypeptide is hydrolyzed approximately 50-fold more rapidly than intact insulin. Similar results have been reported for protease Pi (7). Therefore, it appears possible that dissociation of intact insulin and/or its cleavage products from the enzyme may be a rate-limiting step for degradation of additional insulin molecules. In this regard, the characteristics of low $K_m$ and low $K_{cat}$ are not unlike those of small protein inhibitors of proteases (26), as was noted by Barrett and coworkers (7) for protease Pi.

Protease Ci, like protease Pi (5, 9), is not inhibited by sulfhydryl blocking agents, such as N-ethylmaleimide and iodoaceticamide. This insensitivity of the bacterial enzymes distinguishes them from the human and Drosophila insulin-degrading enzymes that are highly susceptible to inactivation by such reagents (9, 10). The predicted amino acid sequence of protease Pi contains a single Cys residue (27), whereas the human insulin-degrading enzyme has 12 Cys residues (8). One of the Cys residues in the human enzyme is present in the metal-binding site (HXCHH) in the firstly conserved domains of insulin-degrading enzymes (28). Protease Pi also contains the metal-binding motif (HXXGH) but lacks the Cys residue (27). Protease Ci may contain a similar metal-binding site to that of protease Pi, which might explain its lack of sensitivity to sulfhydryl-modifying agents. Isolation and sequence determination of the DNA clone for protease Ci should reveal whether this is true.

Protease Ci is capable of hydrolyzing as many as 10 sites among the total 29 peptide bonds in oxidized insulin B-chain. In contrast, protease Pi cleaves only the Tyr16-Leu17 bond (7), which is also hydrolyzed by protease Ci. Like protease Ci, the insulin-degrading enzymes from Drosophila and rat also hydrolyze multiple peptide bonds in the insulin B-chain (29), although protease Ci is able to cleave many more sites. In addition, all the peptide bonds hydrolyzed by the various eukaryotic insulin-degrading enzymes are also sensitive to degradation by protease Ci, except for the Ser9-His10 bond and the Phe24-Phe25 bond, which are cleaved only by the rat enzyme (29).

Protease Ci appears to preferentially degrade relatively small-sized polypeptides, in contrast with other soluble pro-
teases in the cytoplasm of E. coli, proteases Do (DegP), Re (Tsp), Mi, Fa, So, La (Lon), and Ti (Clp) (1, 2). In addition to oxidized insulin B-chain, protease Ci rapidly hydrolyzed glucagon and degraded lactalbumin and lysozyme, although at much slower rates. However, little or no trichloroacetic acid-soluble products are generated by the purified protease from any protein tested that is larger than 15 kDa. Interestingly, when proteins larger than 15 kDa, such as casein, were incubated with the ATP-dependent protease La or Ti in the presence of protease Ci, the production of acid-soluble products was over 2-fold faster than in its absence. The ATP-dependent proteases appear to catalyze the initial rate-limiting steps in the degradation of highly abnormal polypeptides and of certain normal proteins (3, 4). Therefore, protease Ci may function subsequently in the pathway for intracellular protein breakdown and digest the oligopeptides generated by the ATP-dependent endoproteases.

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