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Subcellular Distribution of Various Proteases in *Escherichia coli*

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It has been reported recently that *Escherichia coli* cells contain eight distinct soluble enzymes capable of degrading proteins to acid-soluble material. Two are metalloproteases that degrade [^{125}I]insulin but not larger proteins: protease Pi, which is identical to protease III, is restricted to the periplasm, and protease Ci is restricted to the cytoplasm. The six others (named Do, Re, Mi, Fa, So, and La, which is the ATP-dependent protease) are serine proteases that degrade [^{14}C]globin and [^{3}H]casein, but not insulin. One of these (Mi) is localized to the periplasm, and one (Re) is distributed equally between the two cellular fractions. The others are present only in the cytoplasm.

Despite our extensive biochemical knowledge about *Escherichia coli*, the proteolytic enzymes in such cells are still poorly defined. The enzymes responsible for the rapid breakdown of highly abnormal proteins (12, 25, 32) and the accelerated degradation of normal cell proteins during starvation (12) have not been identified. Further information about these enzymes is obviously essential for understanding the pathways of intracellular protein breakdown and their regulation (12, 22, 25, 32). In addition, proteolytic enzymes must also play critical roles in other important processes, such as the processing of secretory and membrane proteins (10, 42), utilization of exogenous peptides (22), viral morphogenesis (25), breakdown of colicins (1, 2), and inactivation of certain regulatory proteins (25, 37). An additional practical motivation for characterizing the proteases in *E. coli* is that certain foreign polypeptides (e.g., insulin or interferon) now being cloned in this organism are subject to rapid intracellular degradation (40).

To elucidate the pathways of protein breakdown in *E. coli*, we have begun a systematic study of various proteolytic enzymes in this organism. Recently, we have demonstrated the existence in these cells of eight distinct soluble enzymes capable of degrading intact polypeptides to acid-soluble material (13, 38). The complete purification and characterization of these proteases will be published elsewhere (13; K. H. S. Swamy, C. Chung, and A. L. Goldberg, manuscripts in preparation). Six of these proteases (named Do, Re, Mi, Fa, So, and La) degrade [^{14}C]globin and [^{3}H]casein but not [^{125}I]insulin. These enzymes are all serine proteases that were initially defined by their patterns of elution on ion-exchange chromatography and gel filtration (13, 38; Fig. 1). One of these, protease La, is dependent on ATP for its activity (38), and it is encoded by the *lon* gene (also called *capR* or *deg*) (6, 9). This enzyme therefore appears to be responsible for the rate-limiting steps in protein breakdown in vivo and for the energy requirement of this process (18, 28). Two other proteases (named Pi and Ci) degrade insulin and similar-sized polypeptides (e.g., glucagon or calcitonin), but not globin or casein. These two enzymes are metalloproteases that can be clearly distinguished by chromatography on DEAE-cellulose (13, 38).

These soluble proteases all appear to be new enzymes with the exception of the insulin-degrading activity, protease Pi, which appears to be identical to protease III originally purified by Cheng and Zipser (7), using the amino-terminal fragments of β-galactosidase (auto-α) as a substrate. Several other "proteases" have been reported in *E. coli* (29, 31, 33, 34, 36). Some (36) of these (proteases A, B, and C) appear to be mixtures of proteolytic enzymes (38), whereas others (proteases I and II) (29, 31) have been isolated by using chromagenic substrates for proteases, but have little or no ability to digest intact proteins (17, 29, 30). Furthermore, mutants lacking these enzymes (e.g., proteases I, II, and III) have a normal capacity to degrade cell proteins (8, 14, 17, 23, 24).

One important clue to the functions of the eight soluble proteases could be their subcellular localization. In gram-negative organisms, many hydrolytic enzymes are located in the periplasmic space (15), and an analogy between this region and the degradative organelles of eucary-

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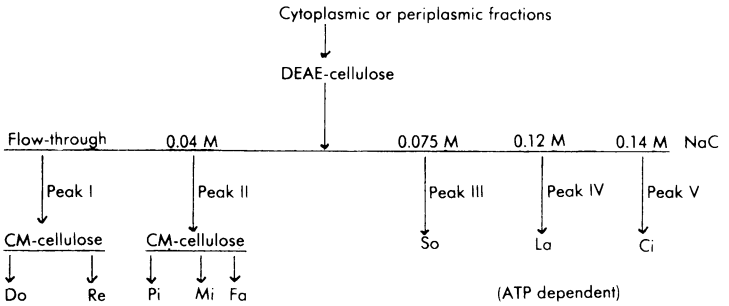

FIG. 1. Summary of the procedures used to isolate the various *E. coli* proteases. Pi and Ci are insulin-degrading proteases; all others degrade globin and casein but not insulin.

otic cells has even been proposed (11). Earlier workers have demonstrated that, upon osmotic shock or conversion to spheroplasts, a large amount (10 to 45%) of the cell's casein-degrading activity is released (3, 17, 35), and the amount of this proteolytic activity in the periplasm appeared to be severalfold higher in cells grown on rich rather than minimal medium (17). Regnier and Thang (35) have shown that multiple peaks of casein-degrading activity exist in both periplasmic and cytoplasmic fractions, but they did not define these activities further. The present studies were undertaken to determine the subcellular location of the two *E. coli* enzymes that degrade insulin and the serine proteases that degrade globin and casein.

MATERIALS AND METHODS

For these studies we used *E. coli* K-12 strain NF172 (*leu-6 thi* Str^r *tonA21* T5^r T6^s *lacY* λ$^-$ F$^-$) (41). The cells were grown to an absorbancy at 550 nm of 2.2 in Luria broth, and the subcellular fractions were prepared by the method of Nossal and Heppel (27). After the osmotic shock step, the cells were suspended in 50 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$–200 mM KCl and were disrupted in a French pressure cell at 14,000 lb/in^2. After removing the unbroken cells by low-speed centrifugation (5,000 rpm, 10 min), the supernatant was centrifuged again at 18,000 rpm for 20 min. The pellet, which represents the membrane fraction, was suspended in 50 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$ and washed twice in the same buffer. The supernatant was spun again at 45,000 rpm for 2 h to separate the ribosomal and cytoplasmic fractions. The ribosomes were suspended in 50 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$ and washed twice in the same buffer.

The proteolytic activity in each fraction was assayed by measuring the degradation of [*methyl*-^{14}C](apo)globin, [*methyl*-^{3}H]casein, and [^{125}I]insulin to acid-soluble material, as reported previously (38). The purity of the subcellular fractions (15) was monitored by assaying for β-galactosidase (43), an exclusively cytoplasmic enzyme, and alkaline phosphatase (20), an exclusively periplasmic enzyme.

The various proteolytic enzymes in the cytoplasmic and periplasmic fractions of *E. coli* were isolated by a combination of ion-exchange chromatographic steps, as described previously (13, 38). The order of procedures used to isolate these eight proteolytic activities is summarized in Fig. 1, and further details are presented in the figure legends. Initial fractionation on DEAE-cellulose yielded five peaks of proteolytic activity, two of which were further resolved by carboxymethyl (CM) cellulose chromatography, as described below and in the figure legends.

RESULTS

The proteolytic activity of various subcellular fractions is shown in Table 1. The insulin-degrading activity was present in both the periplasmic and the cytoplasmic fractions, but not in the ribosomal or membrane fractions. In contrast, globin-hydrolyzing and casein-hydrolyzing activities (Table 1) were found in large amounts not only in the cytoplasm, but also in the membrane and ribosomal fractions. However, it is likely that much of the globin-degrading activity recovered in the ribosomal fraction is actually associated with the small membrane particles that cosediment with the ribosomes (41).

The periplasmic fraction contained about 12% of the total globin-degrading activity, and this activity was never stimulated by ATP, in accord with our earlier observations (26). A slight stimulation (10 to 20%) by ATP was found reproducibly in cytoplasmic, ribosomal, and membrane fractions. Previously, we observed much greater effects of ATP in the soluble (26) and membrane (41) fractions when cells were lysed by more gentle techniques than were used in this experiment (Table 1). In general, casein- and globin-degrading activities showed similar subcellular distributions, although the casein appeared to be especially susceptible to the enzymes released in the stage 1 shock fluid and the periplasmic fraction.

To identify the proteolytic enzymes in periplasm and cytoplasm, these fractions were analyzed on a DEAE-cellulose column as described previously (38). The periplasm contained insulin-degrading activity corresponding to protease

TABLE 1. Subcellular distribution of proteolytic activity in *E. coli*[a]

Subcellular fraction[b]	% Total activity recovered of:		Hydrolyzing activity (U/g of cells)[c] of:				
			^{125}I-insulin	^{14}C-globin		^{3}H-casein	
	Alkaline phosphatase	β-Galactosidase		−ATP	+ATP	−ATP	+ATP
Stage I shock fluid	11.0	0.36	19.0	1.4	1.2	23.6	21.7
Periplasm	78.0	2.5	213.0	10.5	10.7	75.0	74.0
Membrane	0.7	0.54	2.5	26.6	30.0	42.0	49.0
Cytoplasm	9.6	85.0	250.0	33.0	36.0	99.0	109.0
Ribosomes	0.7	11.6	10.0	18.3	21.0	44.0	45.0

[a] *E. coli* NF172 was grown to exponential phase (absorbance at 550 nm of 2.2) in LB broth containing 0.5 mM isopropyl-β-D-thiogalactoside.

[b] Each fraction was assayed for proteolytic activities and for the marker enzymes alkaline phosphatase and β-galactosidase.

[c] One unit of insulin-degrading activity is defined as 1 μg of insulin hydrolyzed in 60 min at 37°C and 1 U of globin- or casein-degrading activity is defined as 1 μg of globin or casein hydrolyzed in 60 min at 30°C.

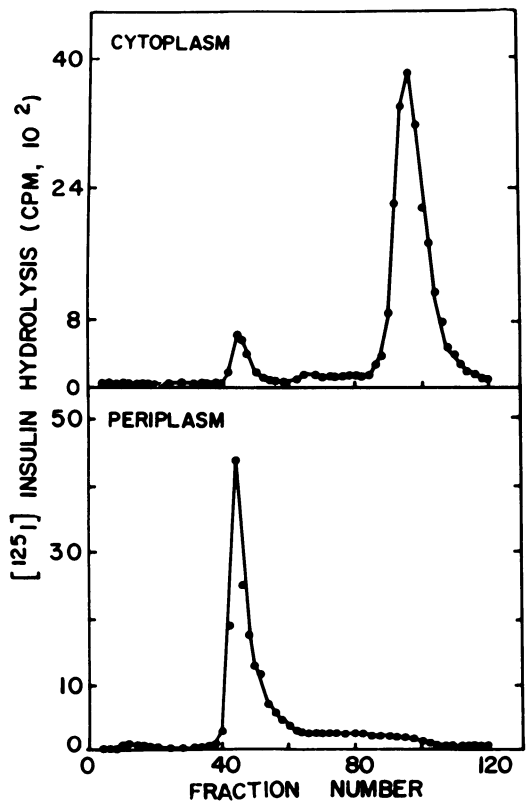

FIG. 2. DEAE-cellulose chromatography of periplasmic and cytoplasmic insulin-degrading activity. The cellular fractions were prepared from *E. coli* NF172 as described in the text. The periplasmic fraction was concentrated by precipitating the proteins by adding solid ammonium sulfate to 95% saturation. After centrifugation at 31,000 × *g* for 30 min, the pellet was dissolved in 10 mM Tris-hydrochloride, pH 7.8, containing 5 mM $MgCl_2$, and both this and the cytoplasmic fraction were dialyzed against the same buffer. The proteases in each fraction were analyzed by chromatography on a DEAE-cellulose column (1.5 by 16 cm) equilibrated with the dialysis buffer. The adsorbed proteins were eluted with 350 ml of a linear salt gradient (0 to 0.25 M NaCl). Alternate fractions were assayed for [^{125}I]insulin-degrading activity.

Pi, but did not show any protease Ci activity (Fig. 2). In contrast, the soluble cytoplasm contained the insulin-hydrolyzing activity corresponding to protease Ci, but only 10% of the total protease Pi activity. Thus, the two insulin-degrading activities are localized to different regions of the cell.

Previous studies (13, 38; C. Chung, K. H. S. Swamy, and A. L. Goldberg, unpublished data) indicated that the periplasmic insulin-degrading activity, protease Pi, closely resembles in physical and chemical properties an enzyme isolated by Cheng and Zipser (7) and named protease III. To test whether protease Pi is in fact identical to protease III, a similar enzyme fractionation on DEAE-cellulose was performed with extracts of *ptr*$^+$ and *ptr*$^-$ strains. The latter mutants (8), which were kindly provided by Edmund Cheng, lack protease III. The *ptr*$^-$ strain also completely lacks the periplasmic insulin-degrading enzyme Pi, but contains normal amounts of the cytoplasmic activity protease Ci (Fig. 3). These data also confirm our earlier conclusion that the two insulin-degrading activities, Pi and Ci, are distinct enzymes.

Chromatography of soluble extracts of *E. coli* on DEAE-cellulose resolved four different peaks or proteolytic activity against globin and casein

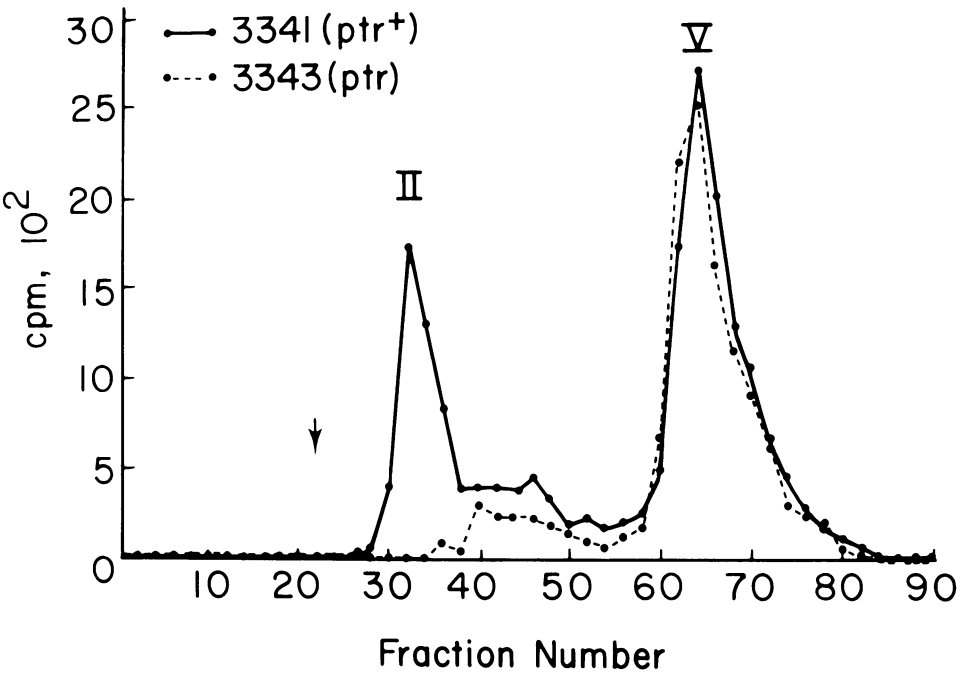

FIG. 3. Analysis of insulin-degrading activity in wild type (●——●) and a protease III-deficient mutant (*ptr*) (●---●) of *E. coli*. Cell-free extracts were prepared as described previously (38) from wild-type (3341) and mutant (3343) strains grown to stationary phase. After dialysis against 10 mM Tris-hydrochloride, pH 7.8, containing 5 mM $MgCl_2$, 150 mg each of protein of wild-type and mutant extracts was applied separately to DEAE-cellulose columns (1.5 by 14 cm). The adsorbed proteins were eluted with a 0 to 0.25 M linear NaCl gradient (arrow indicates the start of the gradient), and alternate fractions were assayed.

which are designated peaks I to IV according to their order of elution (38). Figure 4 compares the elution patterns from DEAE-cellulose of the globin-degrading activities in periplasmic and cytoplasmic fractions. Whereas the globin-degrading activities corresponding to peaks I and II were present in large amounts in both fractions, peaks III and IV, which contain the ATP-stimulated protease La, were found only in the cytoplasmic fraction. After these procedures for subcellular fractionation, we found in peak IV appreciable ATP-independent activity and a much smaller stimulation by ATP than after our usual preparative methods (13, 38). This finding probably resulted from the use in cell fractionation of sucrose and EDTA, which inactivate the ATP-stimulated enzyme (26; unpublished data).

Since both periplasmic and cytoplasmic fractions contained proteolytic activity in peak I, we attempted to determine whether the two proteases within this peak (38), the unusually large enzyme Do (520,000 daltons) and the much smaller Re (68,000 daltons), are distributed similarly in the cell. The peak I activity from each fraction was pooled, dialyzed against 10 mM sodium acetate buffer (pH 5.6), and loaded onto a CM-cellulose column equilibrated with the same buffer. The proteins were eluted with a 0 to 0.3 M NaCl gradient in the same buffer, and the fractions were assayed against globin. About 86% of Do was in the cytoplasm, and the rest was in the periplasm. In contrast, Re was almost equally distributed between these two fractions (Table 2).

Proteolytic activity corresponding to peak II was also found in both periplasmic and cytoplasmic fractions. Peak II contains three proteolytic enzymes, one active against insulin (protease Pi) and two active against globin or casein (proteases Mi and Fa). Whereas the insulin-degrading activity of peak II (Pi) was almost completely periplasmic (Fig. 2; Table 1), the globin-degrading activity was distributed equally between cytoplasm and periplasm (Fig. 4). To determine the relative distribution of the two globin-degrading activities (Mi and Fa), the fractions under peak II were pooled, concentrated by ultrafiltration through a PM10 membrane, dialyzed against 10 mM sodium acetate buffer (pH 5.2) containing 2.5 mM $MgCl_2$, and loaded onto

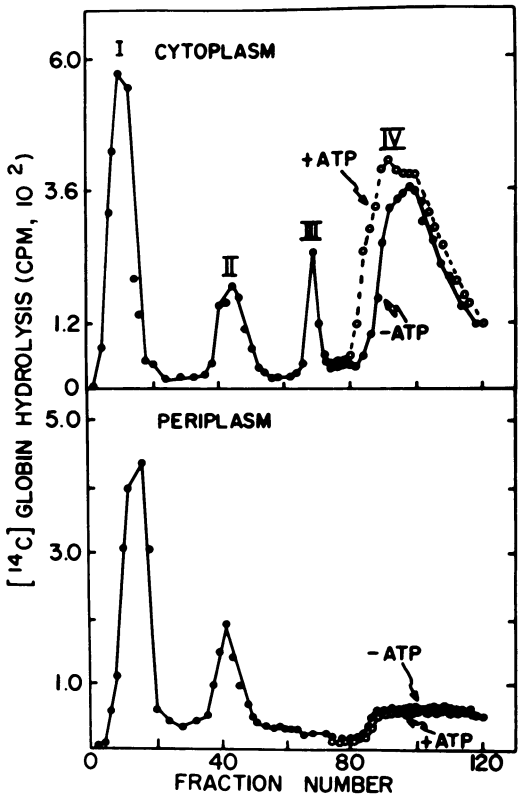

FIG. 4. DEAE-cellulose chromatography of periplasmic and cytoplasmic globin-degrading activity. Experiment was performed as described in the legend to Fig. 2 on those same extracts. Fractions were assayed for [^{14}C]globin degradation. In analogous experiments similar data were obtained when proteolytic activity was measured against [^{3}H]casein.

a CM-cellulose column equilibrated with this buffer. The proteins were eluted with a linear salt gradient (0 to 0.3 M NaCl), and the fractions were assayed for proteolytic activity. Almost all of the activity against [^{14}C]globin of Mi was found in the periplasm, and that of Fa was found in the cytoplasm (Table 2). In these same experiments with CM-cellulose columns, we also confirmed the periplasmic location of the insulin-degrading activity (Pi) as found above (Fig. 2).

The globin-degrading proteases Mi and Fa differ appreciably in their stabilities at 50°C (13, 38). To confirm the localization of these enzymes, we also compared the heat stabilities of the globin-degrading activities in peak II obtained from periplasmic and cytoplasmic fractions (Fig. 4). The peak II enzyme in the periplasm was rapidly inactivated after incubation at 50°C (78% loss within 15 min). This lability is characteristic of protease Mi (13). By contrast, the peak II protease from the cytoplasm was stable under these conditions, as would be expected for protease Fa (13). These results thus confirm the earlier findings with CM-cellulose chromatography (Table 2) that Mi is localized to the periplasm and Ci is localized to the cytoplasm.

DISCUSSION

The finding that the two insulin-degrading proteases are localized to distinct regions of the cell led us (38) to suggest a simple nomenclature for them: Ci for the cytoplasmic insulin-degrading enzyme and Pi to indicate the periplasmic insulin-degrading enzyme, which we showed in Fig. 2 to be identical to the protease III described by Cheng, Zipser, and co-workers (7, 8). Unfortunately, such a simple nomenclature based on subcellular distribution cannot be applied to the globin-degrading activities, whose distribution is more complex. Globin- and casein-hydrolyzing activities were found in all subcellular fractions (Table 1) including mem-

TABLE 2. Subcellular distribution of *E. coli* proteases[a]

Protease	% Total activity recovered in[b]:	
	Periplasm	Cytoplasm
Globin- or casein-degrading enzyme		
Do[c]	16	84
Re[c]	45	55
Mi[c]	100	ND
Fa[c]	ND[d]	100
So	ND	100
La	ND	100
Insulin-degrading enzymes		
Pi[c]	87	13
Ci	ND	100

[a] The periplasmic and ribosome-free cytoplasmic fractions were prepared as described in Table 1. A portion of each fraction was subjected to DEAE-cellulose chromatography and further purification as described previously (39).

[b] To determine the total amount of enzymes in periplasm and cytoplasm, the material under each peak was pooled, and the combined activities were then corrected for the initial volumes of each fraction. The amount of enzyme activity recovered in these fractions is expressed as the percentage of the total of the periplasmic and cytoplasmic activities.

[c] Proteases Do and Re from peak I and Mi and Fa from peak II were separated on CM cellulose as described in the text. The distribution of proteases Pi and Ci was determined after DEAE-cellulose chromatography (Fig. 1). Similar data were obtained in two other experiments.

[d] ND, Not detectable.

branes, in agreement with earlier observations (3, 17, 35). The relationship between these soluble enzymes and the proteases in the membrane and ribosomal fractions remains to be clarified. Proteolytic activity has been reported previously in both the outer (16, 19, 21, 33, 34) and inner (4, 5, 21, 41, 44) membranes, where it may be involved in the processing of secretory and membrane proteins. Unfortunately, the membrane-bound enzymes that hydrolyze globin and casein have not yet been characterized, and at present it is unclear whether any of the six soluble serine proteases also exist in association with the membrane. Since these studies were completed, Regnier (33, 34) has reported the isolation of a casein-degrading protease (named protease IV) from the outer membrane of *E. coli*. Based on its properties (32, 34), this membrane-derived activity appears to be distinct from the eight soluble enzymes.

The differing subcellular localizations of these enzymes suggest distinct physiological functions. The location of protease Pi to the periplasmic space (Fig. 2) may account for the finding (8; C. Chung, K. H. S. Swamy, and A. L. Goldberg, manuscript in preparation) that mutants lacking this enzyme (i.e., *ptr*⁻ cells lacking protease III) have no defect in the degradation of intracellular proteins, including the rapid breakdown of nonsense fragments of β-galactosidase, which are excellent substrates for this enzyme in vitro (7). Presumably, the cytoplasmic insulin-degrading activity plays a role in the degradation of such short intracellular polypeptides or in the hydrolysis of similarly sized polypeptides generated as intermediates during complete degradation of larger cell proteins. Only one casein-degrading protease, Mi, was restricted to the periplasmic space, whereas one (Re) was found in both periplasm and cytoplasm. By contrast, the ATP-dependent protease La, as well as Do, Fa, and So, is strictly cytoplasmic. Presumably, some or all of these latter enzymes, and Re, are involved in the inital steps in breakdown of intracellular proteins.

A primary task for future research will be to clarify which of these eight enzymes are involved in the selective degradation of abnormal proteins (12), in the catabolism of normal cell proteins during starvation (12), and in other proteolytic processes. The localization of proteases Pi, Mi, and Re to the periplasmic region may indicate that they are involved in the processing or degradation of periplasmic or membrane components. It is now well established that such proteins are initially synthesized as larger precursors (10, 42) and are subsequently converted to mature forms by proteolytic enzymes, probably associated with the membrane. It is possible that the periplasmic proteases are also involved in such maturational processing of secreted proteins or in the subsequent degradation of the released amino-terminal sequences ("signal peptides").

Several possible functions have been suggested for periplasmic hydrolases (15, 17), including the digestion of exogenous substrates unable to pass through the inner membrane and digestion of dead cells to make valuable constituents available to surviving members of the population. A role for periplasmic proteases in the hydrolysis of exogenous proteins would be consistent with their induction in media containing a large amount of peptides (17; Chung and Goldberg, unpublished data). In addition, the periplasmic proteases may play a role in protection of the cell against toxic polypeptides in the environment, such as colicins which are rapidly degraded by *E. coli* (1, 2).

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