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Characterization of a Membrane-Associated Serine Protease in *Escherichia coli*

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Three membrane-associated proteolytic activities in *Escherichia coli* were resolved by DEAE-cellulose chromatography from detergent extracts of the total envelope fraction. On the basis of substrate specificity for the hydrolysis of chromogenic amino acid ester substrates, the first two eluting activities were determined previously to be protease V and protease IV, respectively (M. Pacaud, J. Bacteriol. 149:6–14, 1982). The third proteolytic activity eluting from the DEAE-cellulose column was further purified by affinity chromatography on benzamidine-Sepharose 6B. We termed this enzyme protease VI. Protease VI did not hydrolyze any of the chromogenic substrates used in the detection of protease IV and protease V. However, all three enzymes generated acid-soluble fragments from a mixture of *E. coli* membrane proteins which were biosynthetically labeled with radioactive amino acids. The activity of protease VI was sensitive to serine protease inhibitors. Using [^{3}H]diisopropylfluorophosphate as an active-site labeling reagent, we determined that protease VI has an apparent molecular weight of 43,000 in polyacrylamide gels. All three membrane-associated serine proteases were insensitive to inhibition by Ecotin, an endogenous, periplasmic inhibitor of trypsin.

Protein catabolism is recognized as an important physiological process in all cells (6, 7). Previous studies in this laboratory have involved the examination of the intracellular degradation of soluble and membrane-bound proteins in steady-state cultures of *Escherichia coli* (22–24). It was shown in these studies and in similar investigations by others (10, 13) that individual proteins are degraded at heterogeneous rates in growing cells. We are currently focusing our investigations on elucidating the pathway(s) for the degradation of membrane proteins in *E. coli*.

We suggest that a system of proteolytic enzymes external to the cytoplasm may be responsible, at least in part, for the degradation of membrane proteins in *E. coli*. This proposal is supported by the finding that several outer membrane proteins are degraded rapidly (24). Such proteins are not directly accessible to cytoplasmic proteases or intracellular energy pools (i.e., ATP) and would be expected to be degraded initially in an ATP-independent fashion by enzymes located in either the membrane or the periplasmic fraction. Furthermore, isolated whole-membrane fragments have been shown to undergo extensive self-digestion (24), a process presumably mediated by membrane-associated protease activities.

Membrane preparations from *E. coli* have been reported to carry out a variety of specific cleavage reactions in vitro. These reports include the descriptions of the cleavage of colicins Ia, A, and AI to inactive polypeptide fragments (1), the cleavage of plasminogen to plasmin (11), caseinolytic activity (19), nitrate reductase-solubilizing activity (12), and ferric enterobactin receptor-modifying activity (8). Additionally, Pacaud (15, 16) has reported the purification from whole-membrane detergent extracts of two enzymes (protease IV and protease V) which are capable of degrading alkylated, high-molecular-weight cytoplasmic proteins in vitro. Protease IV has also been reported to degrade the signal peptide released by signal peptidase from prolipoprotein during the maturation and export process (9). The potential roles which these activities might play in the turnover of *E. coli* membrane proteins have not been investigated.

In the course of our investigations into the characteristics of the membrane-associated proteases in *E. coli* (17), we identified an additional enzyme, which we have termed protease VI. We report here the characterization of this enzyme and compare it with the previously studied membrane enzymes, proteases IV and V. The determination of the biochemical properties of protease VI is a preliminary step in the elucidation of the in vivo function of this enzyme. Additionally, we examined the effect of the endogenous, periplasmic trypsin inhibitor Ecotin (3) on the activity of membrane proteases IV, V, and VI.

MATERIALS AND METHODS

Materials. The ^{3}H-amino acid mixture (1 mCi/ml) and L-[^{3}H]leucine (58 mCi/mmol) were purchased from ICN Pharmaceuticals Inc. [^{3}H]diisopropylfluorophosphate ([^{3}H]DFP) (4 Ci/mmol) and [^{3}H]formaldehyde (100 mCi/mmol) were purchased from Amersham-Searle and New England Nuclear Corp., Boston, Mass., respectively. The protease inhibitor 3,4-dichloroisocoumarin was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. All other inhibitors and nucleotides and the benzyloxycarbonyl–amino acid–*p*-nitrophenol esters (Cbz-amino acid-ONP) were obtained from Sigma Chemical Co., St. Louis, Mo. All electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, Calif.

Bacterial strain and culture conditions. The *E. coli* K-12 strain A33 (*relA*$^+$ arg trpA) was used in these studies. A basal salts medium containing 9.8 g of Na_2HPO_4, 3.23 g of NaH_2PO_4, 12.75 g of KH_2PO_4, 6.37 g of K_2HPO_4, 0.30 g of $MgSO_4$, and 2.4 g of $(NH_4)_2SO_4$ per liter was supplemented with the required amino acids (60 mg/liter) and succinate (0.5%, wt/vol) as the carbon source. Routine growth of bacteria was carried out in 30-liter batch cultures in a model SS-150 fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.). Aeration was maintained at 30 liters/min with an impeller setting of 400 rpm.

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Cell harvest and membrane isolation. Cells were allowed to grow to late-exponential phase (180 to 200 Klett units) and were harvested by rapid concentration with a Pellicon High Performance Ultrafiltration apparatus (Millipore Corp., Bedford, Mass.). The concentrated culture (approximately 1 liter) was washed in 5 liters of cold buffer A [1 mM Tris, 10 mM $MgCl_2$ (pH 7.4)] in the same apparatus and then pelleted by centrifugation at 5,000 × *g* for 20 min. The cells were resuspended in buffer A and lysed by several passages through a French pressure cell at 18,000 lb/in^2 in the presence of trace amounts of DNase and RNase. The whole-membrane fraction was collected from the crude lysate by centrifugation at 100,000 × *g* for 1 h. All steps were carried out at 0 to 4°C. The crude membrane fraction was extracted with sarcosyl, and the resulting insoluble material was reextracted twice with Emulphogen BC-720 (Sigma) as described by Pacaud (16).

Enzyme purifications. The Emulphogen-soluble membrane extract was used as starting material for the purification of membrane-associated proteases by a modification of the previously described procedure (16). Briefly, after an ammonium sulfate fractionation step, the Emulphogen-soluble material was subjected to DEAE-cellulose (Whatman, Inc., Clifton, N.J.) chromatography with a linear 30 to 400 mM NaCl gradient. The active fractions corresponding to protease IV and protease V were subsequently purified individually by gel permeation high-pressure liquid chromatography on a TSK G3000 column (7.5 by 600 mm) (HPLC Technologies). The high-pressure liquid chromatography column was attached to a Waters high-pressure liquid chromatography system (Millipore Corp., Bedford, Mass.). The column was equilibrated in a buffer containing 10 mM sodium phosphate, 10% (vol/vol) glycerol, 0.05% Emulphogen BC-720, and 1 mM β-mercaptoethanol (pH 7.2). Samples (2 to 4 mg) of partially purified protease IV or protease V equilibrated in the running buffer were resolved on the column at flow rates of 0.4 to 0.5 ml/min.

Protease VI was further purified by affinity chromatography on a benzamidine-Sepharose 6B column (1.5 by 6 cm) (Pharmacia, Inc., Piscataway, N.J.) The pooled active fractions from the DEAE-cellulose column were extensively dialyzed against buffer B (10 mM NaH_2PO_4, 10% [vol/vol] glycerol, 0.3% [vol/vol] Emulphogen, 100 mM NaCl, 1 mM β-mercaptoethanol [pH 7.4]) and loaded onto the affinity column equilibrated with the same buffer. The unadsorbed proteins were washed from the column with buffer B, and then bound material was eluted with a linear gradient formed by mixing equal volumes of buffer B with 10 mM HCl, 500 mM NaCl, 10% glycerol, and 0.1% Emulphogen. The fractions were adjusted to pH 7.4 by the addition of 100 mM NaH_2PO_4 (pH 7.4) and then were assayed for proteolytic activity as described below. The active fractions were pooled and dialyzed against buffer B containing 0.1% Emulphogen.

The endogenous *E. coli* trypsin inhibitor, Ecotin, was purified by the procedure of Chung et al. (3).

Proteolytic and esterolytic activity assays. The hydrolysis of *N*-acyl-amino acid–*p*-nitrophenol ester substrates by the membrane proteases was followed spectrophotometrically at 400 nm by the procedure of Pacaud (15, 16). For the detection of proteolytic activity in column eluates, we used as substrates either [^{3}H]methyl casein labeled by the procedure of Rice and Means (20) or radiolabeled *E. coli* membrane proteins prepared in the following manner. Bacterial cells (1 liter) growing in succinate basal salts medium were labeled with [^{3}H]leucine (final concentration, 1 μCi/ml) or a ^{3}H-amino acid mixture (final concentration, 0.5 μCi/ml) and allowed to grow for an additional 1.5 to 2.0 generations (200 Klett units). The cells were broken, and the whole-membrane fraction was collected as described above. The membrane pellet was suspended in buffer C (10 mM NaH_2PO_4, 0.3% [vol/vol] Emulphogen BC-720, 200 mM NaCl, 2 mM DFP [pH 7.2]) by homogenization in a glass-Teflon tissue grinder, and the proteins were allowed to solubilize at room temperature for 1 h. The insoluble material removed by centrifugation at 100,000 × *g* for 1 h was reextracted in an identical manner. The radioactively labeled Emulphogen-soluble protein mixtures were pooled and dialyzed against buffer C without DFP. This material was used as substrate. Typically, the reactions were performed by adding either 5 to 20 μg of ^{3}H-labeled membrane proteins or 100 μg of [^{3}H]casein to 100- to 400-μl portions of column fractions or crude extracts and bringing them to a final volume of 1.0 ml by the addition of 20 mM sodium phosphate (pH 7.4) or 50 mM Tris hydrochloride (pH 7.8). Proteolytic degradation of the labeled substrates was determined after appropriate time intervals (1 to 5 h) by measuring the amount of radioactivity which became soluble in 10% (vol/vol) trichloroacetic acid (TCA). The TCA-soluble material was separated by centrifugation at 13,000 × *g* for 15 min, and a sample of the supernatant was removed for scintillation counting (18).

Active-site labeling of protease VI. [^{3}H]DFP was used to label specifically the active site of protease VI by a modification of the protocol described by MacGregor et al. (12). The purified enzyme (50 to 100 μg) was mixed with [^{3}H]DFP (8 μM; 22 μCi/ml) and sufficient 20 mM sodium phosphate buffer (pH 7.8) to make a total volume of 1 ml. The reaction was incubated for 3 h at 30°C and was terminated by the addition of 2× solubilization buffer (see below).

PAGE. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was carried out with a 10% (wt/vol) slab gel formulated as specified by O'Farrell (14) for the second dimension of the two-dimensional electrophoresis protocol. Membrane proteins were solubilized by heating the protein extract to 100°C for 3 to 5 min in an equal volume of 2× solubilization buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromphenol blue, 0.125 M Tris hydrochloride [pH 6.8]). Proteins were visualized by staining in Coomassie brilliant blue R-250. Radioactive proteins in polyacrylamide gels were detected by impregnating the gels with En^{3}Hance (New England Nuclear) as specified by the manufacturer. The gels were dried under vacuum and exposed to X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C.

Protein determination. The concentration of protein in crude extracts and column eluates was determined by the method of Bradford (2) by using a commercially available kit (Bio-Rad Laboratories) with bovine serum albumin as a standard.

RESULTS

Separation of proteolytic activities. Our initial studies indicated that the total endogenous proteolytic activity of the *E. coli* envelope fraction increases as growth rate slows (24). We subsequently showed that these findings were not simply the result of an increased concentration of labile protein substrates in slowly growing cells, since the esterolytic activities against model substrates for protease IV and protease V increased two- to threefold when cultures were grown on succinate basal salts rather than on glucose-

containing medium (17). As a result of these findings, cultures were grown with succinate as the sole carbon source in all preparations for enzyme purifications.

The detergent-solubilized membrane extracts were initially eluted from a DEAE-cellulose column by a modification of the procedure of Pacaud (16). The profiles of the esterolytic, caseinolytic, and proteolytic activities from the column are shown in Fig. 1. The first two peaks of enzyme activity eluting from the column hydrolyze a variety of chromogenic amino acid esters (Fig. 1A). We have determined these activities to be crude preparations of the previously reported enzymes protease V and protease IV (15, 16) (in order of their elution from the DEAE-cellulose column). It is noteworthy that the enzyme eluting with the starting buffer (protease V) has caseinolytic activity (Fig. 1B) and an inhibitor profile (17) similar to those of an enzyme described by Regnier (19) as protease IV. Thus, there is an unfortunate confusion in the literature concerning the membrane-associated proteolytic activities. The enzyme designated as protease V by Pacaud (15, 16) appears to be identical to the enzyme called protease IV by Regnier (19). The nomenclature of Pacaud has been more generally accepted (9) and is the system used in this manuscript.

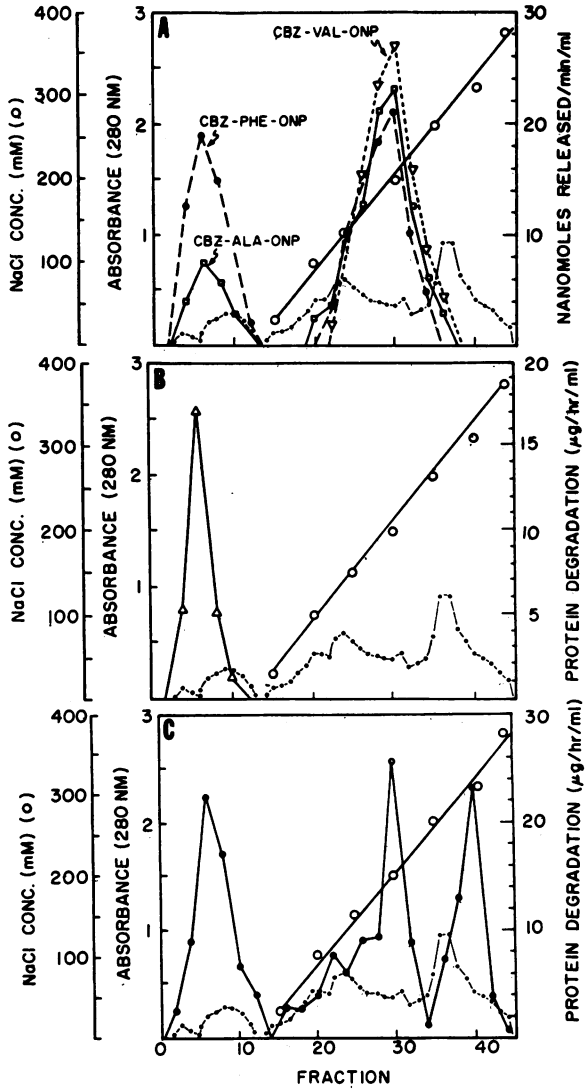

FIG. 1. DEAE-cellulose chromatography of the detergent-solubilized membrane proteases. The column (2.5 by 20 cm) was loaded with 128 mg of protein, and the chromatograph was developed as described in the text. Fractions (5.0 ml each) were collected at a flow rate of 25 ml/h and analyzed for (A) esterolytic activity against Cbz-amino acid-ONP chromogenic substrates (symbols for the specific substrates are as indicated); (B) [^{3}H]methyl casein-degrading activity (△); (C) H-labeled *E. coli* membrane protein-degrading activity (●). Protease activities in panels B and C were determined by the amount of radioactivity released into a 10% (vol/vol) TCA-soluble form at the end of 5 h of digestion. The resulting values were converted to micrograms degraded on the basis of specific activity in the original substrate mixture.

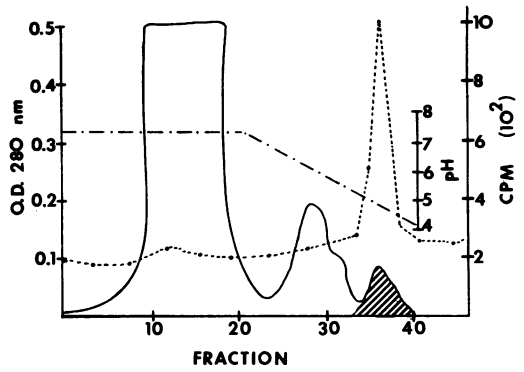

FIG. 2. Affinity chromatography of protease VI on a benzamidine-Sepharose 6B column. The column (1.5 by 6 cm) was loaded with approximately 40 mg of crude protease VI from the DEAE-cellulose column (Fig. 1). The adhering proteins were eluted at a flow rate of 10 ml/h. The fractions (1 ml each) were assayed for protease activity as described in the text. Symbols: ——, A_{280}; - - - -, pH; ● · · · · ●, membrane protein-degrading activity, TCA-soluble cpm. The shaded region indicates peak fractions of protease VI which were pooled.

To identify activities in the column eluates which are capable of hydrolyzing membrane proteins, we used a detergent-solubilized mixture of radiolabeled *E. coli* membrane proteins as substrates. Fractions from the DEAE-cellulose column containing protease IV and protease V were readily detected with this protein substrate mixture (Fig. 1C). Additionally, a third peak of proteolytic activity eluting from the column at a high salt concentration was detected. This activity appears to be another membrane protease, which we have named protease VI. This enzyme did not degrade [^{3}H]casein or the chromogenic substrates Cbz-Phe-ONP, Cbz-Val-ONP, or Cbz-Ala-ONP. An exhaustive search for a chromogenic substrate specific for protease VI was not undertaken. Instead, the membrane protein substrate mixture was used in all subsequent assays for protease VI activity.

Further purification of protease VI. Further purification of protease VI was achieved by affinity chromatography on benzamidine-Sepharose 6B. The column was loaded with approximately 40 mg of protein pooled from active protease VI fractions eluting from the DEAE-cellulose column. The adsorbed proteins were eluted from the affinity column by use of a linear pH gradient (pH 7.4 to 4.0). The peak of protease VI activity (Fig. 2) was detected by its ability to degrade membrane proteins. Since at least three proteolytic activities were present in the initial detergent-soluble membrane extracts, an accurate assessment of the degree of purification was not possible. However, following chromatography on benzamidine-Sepharose, a 43,000-dalton protein was detected which accounted for approximately 25% of the

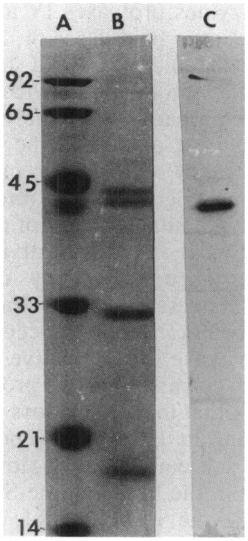

FIG. 3. SDS-PAGE gel separation of affinity-purified protease VI. Approximately 25 μg of protease VI was labeled with [^{3}H]DFP and separated on a SDS-10% polyacrylamide gel. The gel was stained with Coomassie blue R-250 (lanes A and B) and prepared for fluorography. The X-ray film (lane C) was exposed for 3 weeks. Lanes: A, Molecular weight standards (molecular mass in kilodaltons is indicated); B, affinity-purified protease VI; C, fluorograph of lane B.

total protein in the enzyme preparation (Fig. 3). This protein band specifically bound the active-site covalent inhibitor [^{3}H]DFP as determined both by fluorography (Fig. 3) and by cutting and counting gel slices (data not shown). Resolution of [^{3}H]DFP-labeled protease VI by isoelectric focusing and scintillation counting of successive gel slices indicates that this enzyme has a pI of 6.0 (data not shown). When the material from a DEAE-cellulose column was separated on a Sephadex G200 column, the protease VI activity eluted at a position corresponding to the molecular weight of the SDS-denatured monomer (data not shown).

Characterization of protease VI. The activity of protease VI was measured by the release of acid-soluble radioactivity from a complex mixture of labeled *E. coli* membrane proteins. The validity of this assay can be demonstrated by the fact that the release of TCA-soluble radioactivity from the substrate mixture increased in a linear fashion for at least 4 h (Fig. 4). After 4 h, the rate of substrate hydrolysis decreased significantly. The degradation of the radiolabeled proteins was completely inhibited by the inclusion of 2 mM DFP in the reaction mixture. It was also observed that the amount of TCA-soluble radioactivity released after 3 h of incubation in the presence of affinity-purified protease VI was directly proportional to the amount of enzyme added (at enzyme concentrations from 10 to 100 μg/ml) (data not shown).

The thermal inactivation kinetics of protease VI proceeded as first-order reactions (Fig. 5). The enzyme was stable at room temperature or 37°C for 2 h. However, incubation of protease VI at either 45 or 60°C resulted in a rapid loss of activity; the half times of inactivation were 15 min at 45°C and 6 min at 60°C. The linearity of the thermal inactivation plots also indicates that only a single protease was present in the affinity-purified preparation.

Consistent with previous indications (Fig. 3 and 4) that

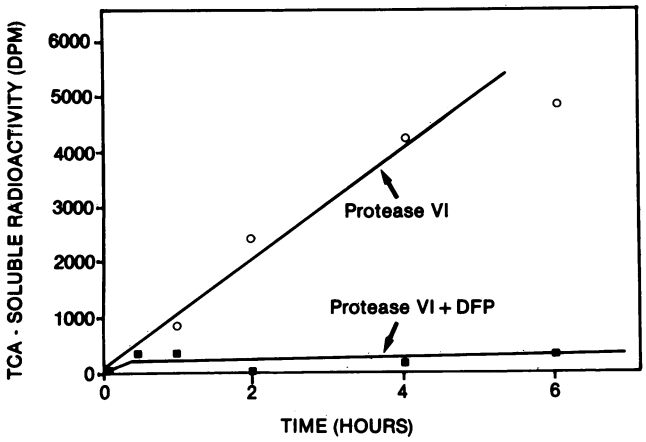

FIG. 4. Degradation of radiolabeled membrane proteins by protease VI. Affinity-purified protease VI (100 μg) was added to a reaction mixture containing 5 μg of ^{3}H-labeled membrane proteins (8,300 dpm/μg) in 50 mM Tris hydrochloride buffer (pH 7.8). The release of TCA-soluble radioactivity over time was measured in the presence (■) and absence (○) of 2 mM DFP.

protease VI is a serine protease, analysis of the pH profile of this enzyme shows that it is maximally active at pH 8.0.

In addition to being sensitive to inhibition by DFP, protease VI activity was inhibited by a variety of other serine protease inhibitors. Table 1 lists the inhibitors, cations, and physiologically important compounds tested for their ability to either inhibit or stimulate the degradation of the membrane protein substrate mixture by protease VI. Significantly, protease VI, unlike proteases IV and V (16), was sensitive to inhibition by benzamidine and *p*-aminobenzamidine. This property was exploited in the affinity

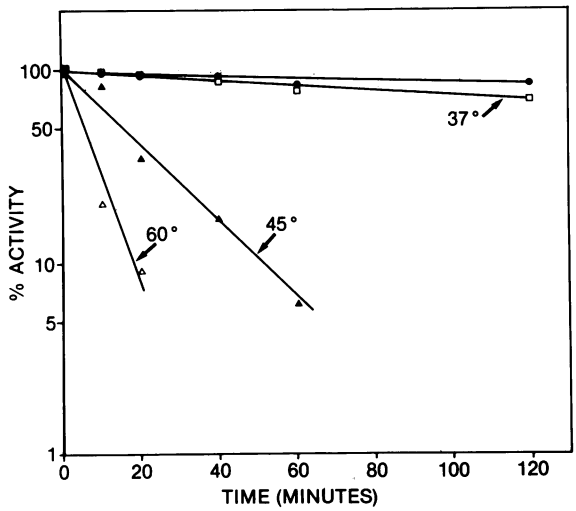

FIG. 5. Thermal inactivation of protease VI. Samples containing approximately 25 μg of affinity-purified protease VI were preincubated for various times at the indicated temperatures. Enzyme activity was measured at room temperature by using the ^{3}H-labeled membrane protein mixture described in Fig. 4. The assays were terminated at 3 h, and the amount of TCA-soluble radioactivity released was compared with the amount released from the untreated enzyme preparation (100% activity = 4.9 μg of substrate released per h per mg of protease VI). Symbols: ●, room temperature; □, 37°C; ▲, 45°C; △, 60°C.

purification step for this enzyme. The degree of inhibition by the compounds phenylmethylsulfonyl fluoride, *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone, and N-α-*p*-tosyl-L-lysine chloromethyl ketone was virtually identical to what we had previously observed with less purified preparations of protease VI (17). Compounds such as ATP (with 1 mM Mg^{2+}), ADP, or AMP had no effect on the activity of affinity-purified protease VI. Inhibitors of cysteine proteases (*N*-ethylmaleimide or iodoacetate), inhibitors of metalloproteases (EDTA and 1,10-phenanthroline), and various cations did not significantly affect the enzyme activity.

Effects of Ecotin on membrane protease activity. Ecotin is a low-molecular-weight periplasmic protein and a potent inhibitor of trypsin and other pancreatic proteases (3). Since this inhibitor would have access to proteases in the *E. coli* membrane fraction in vivo, we examined its effect on the activity of all three membrane-associated proteases. We purified Ecotin to near homogeneity by the published protocol (3). The purified inhibitor exhibited a monomeric molecular weight, pI, and inhibition kinetics with trypsin essentially identical to those previously reported (3) (data not shown). When excess Ecotin was incubated with high-pressure liquid chromatography-purified protease IV or protease V or with affinity-purified protease VI, there was no detectable loss of enzyme activity.

DISCUSSION

The studies reported here have focused on the partial purification and characterization of a newly detected membrane-associated protease which we have termed protease VI. This enzyme differs from the previously reported membrane-associated enzymes proteases IV and V (15, 16) and from the signal and leader peptidases (4, 25, 26).

TABLE 1. Measurements of protease VI activity in the presence of various inhibitors or activators[a]

Inhibitor or activator[b]	Concn (mM)	% Activity[c]
Inhibitors		
None		100
N-ethylmaleimide	5	89
PMSF	5	19
3,4-Dichloroisocoumarin	1	76
TLCK	5	31
TPCK	5	95
Benzamidine	5	44
p-Aminobenzamidine	5	19
DFP	2	17
Iodoacetate	5	83
EDTA	5	91
1,10-Phenanthroline	5	89
Activator or cation		
None		100
ATP/Mg^{2+}	1	98
ADP	1	101
AMP	1	91
Zn^{2+}	10	100
Mn^{2+}	10	91
Ca^{2+}	10	85
Mg^{2+}	10	97

[a] Affinity-purified protease VI (50 μg) was added to each reaction mixture containing 5 μg of ^{3}H-labeled membrane proteins in 50 mM Tris hydrochloride buffer (pH 7.8) in the presence or absence of various inhibitors. The reactions were terminated at 3 h.

[b] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TLCK, N-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, N-α-*p*-tosyl-L-phenylalanine chloromethyl ketone.

[c] Measurements of protease VI activity in the presence of inhibitors are the average of duplicate assays from two separate experiments. Measurements in the presence of activators or cations are determined from duplicate assays in a single experiment.

The purification scheme described here results in a marked increase in the relative abundance of protease VI in the affinity-purified preparations. The resulting enzyme preparation is capable of degrading the radiolabeled substrate mixture at a rate of several micrograms per hour per milligram of enzyme (Fig. 4). This rate is probably an underestimate of the potential activity of protease VI, since we have observed that the specific activity of the enzyme fractions following chromatography on benzamidine-Sepharose was only moderately higher than in the detergent extracts of whole membranes. This occurred even though there was a large increase in the relative abundance of the 43,000-dalton protein corresponding to protease VI (data not shown). It is possible that the activity loss of affinity-purified protease VI resulted from the harsh and potentially denaturing conditions (high salt concentration, low pH) required to elute the enzyme from the benzamidine-Sepharose column. In addition, the enzyme is susceptible to self-digestion. Storage under liquid nitrogen is required to maintain activity. It should be noted that in detergent-solubilized extracts, the activity of protease VI in degrading the substrate mixture was similar in magnitude to those found for proteases IV and V (Fig. 1C).

The inhibitor profile of protease VI (Table 1) is distinct from that found for proteases IV and V (16), which supports the contention that it is another membrane protease. The fact that protease VI is not stimulated by ATP (and Mg^{2+}) and is only moderately inhibited by 3,4-dichloroisocoumarin indicates that this enzyme is also distinct from the ATP-stimulated membrane protease activity reported recently by Klemes et al. (Y. Klemes, R. W. Voellmy, and A. L. Goldberg, Fed. Proc. **45**:1598, 1986). The binding of protease VI to both DEAE-cellulose and benzamidine-Sepharose is similar to the characteristics observed for a membrane-bound enzyme of similar molecular weight called protein *a* or OmpT (5, 21), which is responsible for the modification of ferric enterobactin receptor (8). However, Hollifield et al. (8) report that this enzyme is insensitive to inhibition by DFP or phenylmethylsulfonyl fluoride which would distinguish it from protease VI.

The use of a complex protein mixture as substrate for assaying protease VI activity is a novel and valid approach for identifying proteolytic enzymes of unknown specificity. By presenting a large number of potential substrates (membrane proteins) to the enzyme preparation, one removes the arbitrariness in the choice of substrate (such as using [^{3}H]casein on various chromogenic ester substrates). We have tested protease VI with several chromogenic substrates and found none to be hydrolyzed by this enzyme. The substrates we tested include blocked amino acid esters of valine, phenylalanine, alanine, glycine, glycyl-glycine, and arginine. The screening of additional chromogenic or fluorogenic substrates may yet identify a suitable model substrate for protease VI.

In preliminary experiments, we have examined the components in the membrane protein substrate mixture which are degraded by protease VI. When the mixture is analyzed by two-dimensional gel electrophoresis, a limited number of proteins can be shown to be hydrolyzed by protease VI (manuscript in preparation). In similar studies, we have observed that protease IV and protease V also degrade specific proteins in the membrane-substrate mixture. Furthermore, distinct subsets of the proteins are degraded by each membrane-associated enzyme (manuscript in prepara-

tion). The detergent solubilization process used to extract the radiolabeled membranes results in an artificial substrate mixture containing both cytoplasmic membrane and outer membrane proteins. Since these in vitro substrates may not normally be exposed to the proteases in vivo, it is not possible to determine whether the specific protein components degraded by the enzymes represent in vivo substrates.

The endogenous trypsin inhibitor Ecotin was not observed to affect the rate or extent of substrate hydrolysis by protease VI or by the other membrane-associated enzymes proteases IV and V. The lack of inhibition of membrane protease activity by Ecotin supports the physiological role of Ecotin proposed by Chung et al. (3). Since these workers showed that Ecotin does not inhibit any of the known cytoplasmic or periplasmic proteases, they suggested that this inhibitor functions in protecting *E. coli* from damage by proteolytic enzymes in the gut, its natural habitat. Such a putative protective function for Ecotin is a simple, albeit not readily testable, model. It still remains possible that Ecotin serves a regulatory function in *E. coli* by modulating the activity of other, as yet unidentified, periplasmic or membrane-bound proteases or other serine-type hydrolases such as a phospholipase or a penicillin-binding protein. Clearly, mutants defective in Ecotin are needed to unravel the specific role of this inhibitor.

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