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Role and Location of "Protease I" from *Escherichia coli*

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Pacaud and Uriel described an enzyme from *Escherichia coli* ("protease I") that hydrolyzes acetyl phenylalanine naphthyl ester (APNE). We examined the possible involvement of this enzyme in intracellular protein degradation, its subcellular distribution, and its proteolytic activity. Although the APNE-hydrolyzing activity is localized primarily in the periplasm, proteolytic activity against casein was found in the periplasm, membrane, and cytoplasm with similar specific activities. The APNE-hydrolyzing enzyme did not appear to contribute to the proteolytic activity of the periplasm. A mutant deficient in APNE-hydrolyzing activity lacked all activity in the periplasm but showed a slight percentage of residual activity in the cytoplasm. Extracts of such cells were normal in their ability to hydrolyze casein. The mutant was indistinguishable from wild-type cells in its rate of protein degradation during growth or glucose starvation and in the ability to rapidly degrade puromycin-containing polypeptides. Nitrogen starvation, which increased protein breakdown severalfold, affected neither the total amount nor the distribution of APNE-hydrolyzing activity. The mutant showed no defect in its ability to cleave small phenylalanine-containing peptides released during protein degradation. The mutant and wild-type cells are equally able to hydrolyze exogenously supplied phenylalanyl peptides. These experiments suggest that the APNE-hydrolyzing enzyme is not required for protein degradation and that "protease I" is probably not a protease.

Although intracellular protein degradation was first demonstrated in *Escherichia coli* over 20 years ago (13) and occurs in all cells (7), little is known about the proteolytic enzymes that are involved in this process. Pacaud and Uriel (17) purified and characterized an enzyme from *E. coli* that hydrolyzes acetyl phenylalanine naphthyl ester (APNE), a synthetic substrate of chymotrypsin. This enzyme was reported to hydrolyze *E. coli*, ribonucleic acid polymerase (10), and polynucleotide phosphorylase (21) in vitro, although high enzyme concentrations and long incubations were required. Since no protease had been isolated previously from *E. coli*, this enzyme was designated "protease I." To test for a possible role of this enzyme in protein catabolism, we examined protein breakdown in a mutant deficient in the APNE-hydrolyzing activity. In addition, we compared the intracellular location of this activity and that of proteolytic activity against casein.

MATERIALS AND METHODS

Assays. Protein was determined by the method of Lowry et al. (12). β-Galactosidase was determined by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (23). For such determinations, cells were initially grown in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside.

Proteolytic activity was assayed by the release of free amino groups from casein. The various subcellular fractions were dialyzed against 0.2 M sodium borate, (pH 9.0) to remove tris(hydroxymethyl)-aminomethane (Tris), amino acids, and small peptides (which react with Fluram [see below]). Assay tubes contained 1 ml of the extract (less than 0.5 mg of protein per ml) and 0.33 ml of 2% (wt/vol) casein in borate buffer. Since proteins in the extract may compete with casein as a substrate for proteases, the exogenous substrate was added at 10- to 25-fold greater protein concentrations than that of the extract to minimize this effect. In addition, the autolytic activity in the absence of added casein was determined in parallel incubations, and this value was subtracted from activity measured in the presence of casein. Under these conditions the proteolytic activity against casein was 3- to 10-fold greater than autolytic activity.

Before and after 2-h incubation at 37°C, samples were mixed with equal volumes of cold 10% (wt/vol) trichloroacetic acid to precipitate proteins. After incubation for 1 h at 4°C, the tubes were centrifuged for 10 min at 1,500 × *g*. A 100-μl volume of the supernatant fluid was mixed, with a Vortex mixer, with 1.4 ml of Fluram (Roche Diagnostics; 30 mg/ml) dissolved in acetone. L-Leucine was used as a stan-

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dard. Relative fluorescence at 472 nm was measured on an Aminco-Bowman fluorimeter, after excitation at 390 nm. One unit of casein-hydrolyzing activity was the amount of extract that released in 1 h (in a 100-μl sample) amino groups equal to 1 nmol of leucine. With this assay, proteolytic activity against casein was proportional to the concentration of the extract and was linear for 2 h.

Histone hydrolysis was measured by the appearance of radioactivity from [^{125}I]histone in a form soluble in 15% trichloroacetic acid and 2.5% (wt/vol) silicotungstic acid. This mixture was found to precipitate quantitatively [^{125}I]histone, which is partially soluble in trichloroacetic acid. Histone type IIA (Sigma Chemical Co.) was iodinated by the method of Hunter (11) to 10^5 cpm/μg. The extract (0.1 ml) was incubated with 1 μg of [^{125}I]histone in 1 ml of 50 mM Tris-hydrochloride (pH 8.2) for 1 h at 37°C. Samples removed before and after incubation were mixed with an equal volume of trichloroacetic acid-silicotungstate, and 250 μg of unlabeled histone was added to aid precipitation. The precipitate was removed by centrifugation, and the amount of radioactivity in the supernatant was determined by liquid scintillation counting. When commercial trypsin (Miles Laboratories, Inc., grade V) was used as the protease, the radioactivity released was directly proportional to the incubation time and to the trypsin concentration. However, when crude cell extracts were tested, the activity was not proportional to extract concentration, possibly because of competition between proteins in the extract (about 0.5 mg/ml) and [^{125}I]histone (1 μg/ml). Consequently, all assays were performed with equal protein concentrations.

Hydrolysis of APNE was measured by a modification of the method of Ravin et al. (20). All steps and reagents were at 4°C until addition of trichloroacetic acid. A 2.0-ml volume of substrate was added to 0.5 ml of barbital buffer (0.1 M sodium barbital [pH 7.8], 20% [wt/vol] methanol) containing the dialyzed extract. Substrate was prepared fresh by the addition of 5 ml of APNE (2 mg/ml in acetone) to 95 ml of barbital buffer. After 1 h of incubation, 0.5 ml of Fast Blue Salt B (Matheson, Coleman & Bell) was added while mixing with a Vortex mixer. After 5 min, 0.5 ml of 80% (wt/vol) trichloroacetic acid was added to stop the reaction. Samples were extracted with 5 ml of ethyl acetate. After brief centrifugation, the upper (organic) phase containing the colored naphthol derivative was measured in a Klett colorimeter with a green filter. Standards contained 0 to 500 ng of bovine chymotrypsin (Miles-Seravac). One unit of activity equaled that amount of extract giving the same amount of hydrolysis in 1 h as 25 ng of chymotrypsin. Purified APNE-hydrolyzing enzyme was kindly provided by M. Pacaud and J. Uriel.

Degradation of labeled cell proteins was determined by measuring the appearance of radioactive amino acids in trichloroacetic acid-soluble form as a function of time (6, 8, 19). The hydrolysis of puromycin-containing polypeptides (6) and of normal cell proteins (19) was measured as described previously. Protein degradation was expressed as the percentage of acid-soluble radioactivity relative to that originally present in acid-precipitable material.

^{14}C-labeled phenylalanine, present either as free [1-^{14}C]phenylalanine or in peptides terminating with [1-^{14}C]phenylalanine, was measured by decarboxylation, using an adaptation of the technique of Chang and Fenton (4). $^{14}CO_2$ was collected as described by Odessey and Goldberg (15). Cells were grown in the presence of [1-^{14}C]phenylalanine to label proteins and peptides released by protein degradation. The cells in log phase were then washed and suspended in fresh media containing unlabeled phenylalanine for measurement of protein degradation. Samples (1 ml each) were added to 0.25 ml of 17% (wt/vol) chloramine-T (4) and 10 μl of 10 mM sodium bicarbonate (to serve as CO_2 carrier) in a 25-ml Erlenmeyer flask. $^{14}CO_2$ was collected in a well containing 0.3 ml of phenethylamine. H_2SO_4 (0.5 ml, 1 N) was injected through the rubber stopper into the sample to drive off the CO_2, and the flask was shaken at 20°C for 1 h. The solution in the well was mixed with 4.0 ml of 2,5-bis-2-(5-*tert*-butylbenzoxazoyl)thiophene (Packard Instrument Co., Inc.) (2.5 g per 625 ml of toluene plus 375 ml of absolute ethanol), and radioactivity was determined by scintillation counting.

Bacterial strains and media. *E. coli* K-12 strain A33, requiring arginine and tryptophan, was obtained from B. Davis. Tryptone agar plates used for the isolation of Pr$^-$ mutants contained 1.2% agar (Difco Laboratories), 1.0% tryptone (Difco), and 0.5% NaCl. For other experiments, strains were grown with aeration in Penassay broth (Difco), medium B (14) supplemented with 0.6% glycerol, or glycerol minimal medium (5). M9 minimal medium (1) was used for dipeptide utilization studies. Phenylalanylphenylalanine was obtained from Schwarz/Mann and was checked for homogeneity by chromatography. Free phenylalanine, if present, was at a level of less than about 2%. Phenylalanyl tyrosine, phenylalanyl alanine, phenylalanyl-methyl ester, and phenylalanyl-ethyl ester were kindly supplied by Elkin Blout.

Isolation of mutants deficient in APNE-hydrolyzing activity. *E. coli* mutants deficient in APNE-hydrolyzing activity were isolated by a modified replica plating technique (described below) whereby colonies are imprinted onto filter paper, which is then stained with a chromogenic reagent. The method is simpler and more convenient than the usual spraying or indicator plate techniques and is more sensitive because of enhanced color contrast on the white background.

A culture of *E. coli* BB was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as described by Adelberg et al. (2), and spread on fresh tryptone-agar plates to give about 300 colonies per plate. When the colonies reached a diameter of about 2 mm, a disk of Whatman no. 1 filter paper, slightly smaller than the plate, was applied to the moist agar surface and carefully removed so as to avoid distortion of the colony pattern. After drying, the paper was immersed in a staining solution prepared immediately before use by mixing 20.0 ml of 0.05 M Tris-hydrochloride (pH 7.5) containing 40 mg

of Fast Blue Salt B with 6.0 ml of dioxane containing 20 mg of APNE. The disk was blotted to remove excess solution and allowed to dry at room temperature. Within 5 min, colony imprints with APNE-hydrolyzing activity became visibly purple, reaching maximum intensity in about 30 min. Imprints of colonies deficient in this activity appear as faint yellow spots, the position of which serves to locate the colony residue on the master plate. With efficient mutagenic treatment, the mutant frequency is about 10^{-3}. Mutants so obtained are called Pr^-; the isolate used in this study is designated Pr_8^-.

Preparation of subcellular fractions. *E. coli* in logarithmic phase were collected, and subcellular fractions were prepared by a modification of the osmotic shock method of Nossal and Heppel (14). A 0.5-liter cell suspension at approximately 100 Klett units (about 100 mg of protein, total) was collected by centrifugation for 10 min at 10,000 × *g*, washed twice in a solution of 10 mM Tris-hydrochloride (pH 7.1 or 8.1) and 30 mM NaCl and suspended in 20 ml of 33 mM Tris-hydrochloride. A 20-ml volume of 33 mM Tris-hydrochloride–40% (wt/vol) sucrose was added, and ethylenediaminetetraacetate (EDTA) was added to a final concentration of 0.1 or 1.0 mM. Cells were shaken gently for 10 min at room temperature and were pelleted for 10 min at 13,000 × *g*. The supernatant was designated "stage I shock fluid" (13). The pellet was drained, immediately suspended in 30 ml of ice-cold water or 2 mM $MgCl_2$, and shaken gently for 10 min at 4°C. Cells were then centrifuged at 11,000 × *g* for 10 min, and Tris-hydrochloride (1 M, pH 7.1) was added to the supernatant fluid to give a final concentration of 10 mM (pH 7.1). The supernatant fluid (Heppel's stage II shock fluid) was designated the "periplasmic" fraction. Cells were resuspended and sonically oscillated in 5 ml of the same buffer. After removal of unbroken cells by centrifugation, the membrane fraction was prepared by centrfiguation for 10 min at 30,000 × *g*, and the pellet was resuspended in 10 mM Tris-hydrochloride (pH 7.1). The 30,000 × *g* supernatant fluid was designated the "cytoplasmic" fraction; the pellet was designated the "membrane" fraction. Samples of each fraction were dialyzed for 24 h against borate buffer, and all fractions were stored at −30°C.

RESULTS

Subcellular fractions were prepared by osmotic shock and assayed for APNE-hydrolyzing activity. In both *E. coli* K-12 and *E. coli* BB (data for BB not shown), the activity was found primarily in the periplasmic fraction (Table 1). This fraction contained from 1/2 to 2/3 of the total activity, depending on the strain and growth medium, and its specific activity was 15- to 20-fold higher in the periplasm than in cytoplasmic or membrane fractions. By comparison, the periplasm contained from 10 to 50%, the cytoplasm contained from 50 to 80%, and the membrane contained from 5 to 15% of the total proteolytic activity, depending on the strain and growth conditions (Table 2). The specific activity of proteolytic activity was highest in the periplasm (or stage I shock fluid in one case), ranging between two- and fourfold that of other fractions. Cell lysis was not responsible for release of APNE-hydrolyzing activity or proteolytic activity into the periplasmic fraction, since more than 90% of the β-galactosidase, an intracellular enzyme, remained with the cytoplasmic fraction. These fractions were also active in the hydrolysis of histone (data not shown) as well as casein. The membrane fraction retained hydrolytic activity against histone after several washings with buffer. In addition, all three fractions were able to hydrolyze endogenous proteins, since significant proteolysis was measured in each fraction in the absence of added casein. In all of these experiments, proteolysis by any subcellular fraction clearly may represent the action of more than one hydrolytic enzyme.

The amount of proteolytic activity released in the periplasm depended on the medium in

TABLE 1. *Distribution of APNE-hydrolyzing activity in subcellular fractions of E. coli*[a]

Growth medium	Fraction	Protein (% total)	β-Galactosidase (% total)	APNE-hydrolyzing activity		
				U	Sp act (U/mg)	% Total
Penassay broth	Stage I shock fluid	4	1	20	7.1	16
	Periplasm	16.5	7	81	6.9	66
	Cytoplasm	70.5	90	21	0.42	17
	Membrane	9	2	1.5	0.20	1
Medium B + glycerol	Stage I shock fluid	3	0	6	2.7	8
	Periplasm	5	0.5	41	10.8	51
	Cytoplasm	68	98	29	0.58	36
	Membrane	24	1	4	0.22	5

[a] Strain A33 in log phase was treated with sucrose-0.1 mM EDTA (pH 7.1) (stage I). At stage II, cells were shocked with 2 mM $MgCl_2$.

TABLE 2. *Distribution of proteolytic activity against casein in subcellular fractions of E. coli*[a]

Expt	Growth medium	Fraction	Proteolytic activity against casein		
			U	Sp act (U/mg of protein)	% Total
1	Penassay broth	Stage I shock fluid	0	0	0
		Periplasm	77	3.28	46
		Cytoplasm	79	0.79	47
		Membrane	11	0.86	7
	Medium B + glycerol	Stage I shock fluid	4	1.7	3
		Periplasm	11	3.0	10
		Cytoplasm	88	1.8	75
		Membrane	14	0.8	12
2	Penassay broth	Stage I shock fluid	18	3.2	13
		Periplasm	44	4.4	31
		Cytoplasm	68	0.9	49
		Membrane	8.4	1.2	6
	Medium B + glycerol	Stage I shock fluid	11.5	23.0	6
		Periplasm	22	3.4	12
		Cytoplasm	154	2.4	80
		Membrane	4.6	0.5	2

[a] Strain A33, harvested in log phase, was treated with sucrose-0.1 mM EDTA (pH 7.1) (stage I). At stage II, cells were shocked with 2 mM $MgCl_2$.

which the cells were grown (Table 2). Cells grown on either Penassay broth or medium B plus glycerol were subjected to osmotic shock in parallel. Yet the Penassay broth-grown cells released more proteolytic activity (46 and 44% in the combined stage I and stage II shock fluids) than medium B-grown cells (13 and 18%). Comparison of protein- and APNE-hydrolyzing activity released in these experiments showed that cells grown on Penassay broth released 20.5% of the total protein in the shock fluids as compared to 8% for cells grown on medium B (Table 1). Similar results were obtained in other experiments. The cells grown on Penassay broth released 82% of the APNE-hydrolyzing activity versus 59% for the medium B-grown cells (Table 1). Cell lysis can account for only a small fraction of these differences since lysis (measured by release of β-galactosidase) was 8 and 9% for the Penassay broth-grown cells versus 0.5 and 9% for the medium B-grown cells.

Nitrogen deprivation is known to increase protein degradation severalfold in *E. coli* (13, 19). We tested to see whether nitrogen starvation leads to an increase in either APNE-hydrolyzing or proteolytic activity. Deprivation of nitrogen for 2 h (during which time the viable count remained constant) did not affect either the level or the subcellular distribution of APNE-hydrolyzing activity or of proteolytic activity against casein (data not shown).

The periplasmic fraction thus contained both proteolytic activity and the APNE-hydrolyzing activity. To test whether the APNE-hydrolyzing enzyme contributed to the proteolytic activity of the periplasmic fraction, we studied the mutant deficient in APNE-hydrolyzing activity (Pr^-). In three separate experiments, the mutant was found to contain 3, 2, and 10% of the total APNE-hydrolyzing activity of the wild-type parent strain. In all cases, however, the mutant had no detectable periplasmic APNE-hydrolyzing activity; the residual activity of the mutant was all in the cytoplasmic fraction (Table 3). When the mutant and wild type were examined for proteolytic activity, no significant differences were found in any of the subcellular fractions (Table 4). Thus, APNE-hydrolyzing activity does not appear to contribute significantly to the proteolytic activity of the periplasm measured at pH 9.0 with casein as a substrate.

Further experiments also failed to correlate proteolytic activity with APNE hydrolysis. The casein-degrading activity of the periplasm was highly sensitive to heat and was inhibited by EDTA, in contrast to APNE-hydrolyzing activity (Table 5). Furthermore, the pH profiles of the two activities differed. Histone hydrolysis

showed a pH optimum of 8 to 9 with very little activity below pH 7.0, whereas APNE hydrolysis had a pH optimum of 7.6 with 75% activity at pH 6.0.

We also compared the pure bacterial enzyme (17) and chymotrypsin with respect to their esterase activity against APNE and their proteolytic activity (Fig. 1). The specific activity of the bacterial enzyme against APNE was 10% that of chymotrypsin in accord with the findings of Pacaud and Uriel. However, proteolytic activity against casein was detectable at 37°C with as little as 25 ng of chymotrypsin per ml but was undetectable with 12,500 ng of APNE-hydrolyzing enzyme per ml. Together these experiments argue against the APNE-hydrolyzing enzyme from *E. coli* being a protease.

Although this enzyme does not contribute measurably to the proteolytic activity of the cell against casein, it is still possible that the APNE-hydrolyzing enzyme plays some role in protein degradation. If the APNE-hydrolyzing enzyme is required in protein degradation, this process should be reduced severely in the Pr^- mutant. Several experiments failed to demonstrate such a defect (Fig. 2 and 3). Measurements of protein degradation rates during growth showed no difference between Pr^+ and Pr^- strains. For example, in typical experiments (data not shown), in Pr^+ and Pr^- 3.3 and 3.2%, respectively, of the proteins labeled during a 5-min exposure to [^{3}H]leucine were degraded in 1 h (see also Fig. 2, controls). Starvation for a carbon source (8) increased degradation of normal proteins severalfold. The Pr^- mutant was just as active as the wild type in protein degradation under such conditions (Fig. 2B). Another possible role for the APNE-hydrolyzing activity might be in the rapid degradation of abnormal proteins, such as the polypeptides released prematurely from the ribosome as a result of incorporation of puromycin (6, 18). The proteolytic system responsible for this process may be distinct from that responsible for breakdown of normal proteins during starva-

TABLE 3. *APNE-hydrolyzing activity of the Pr^- mutant*[a]

Strain	APNE-hydrolyzing activity (total units) in the fraction	
	Periplasm	Cytoplasm
Expt 1		
Pr^+	78.5	14.2
Pr^-	0	8.7
Expt 2		
Pr^+	50.0	30.8
Pr^-	0	1.40

[a] Strains Pr^+ and Pr^- were grown on Penassay broth and harvested in log phase, and subcellular fractions were prepared as described in the text. Sucrose-5 mM EDTA treatment was performed at pH 8.1 prior to osmotic shock with distilled water.

TABLE 5. *Effects of heat and EDTA on APNE and casein hydrolysis*[a]

Treatment	APNE hydrolysis (%)		Casein hydrolysis by periplasm (%)
	Pure enzyme	Periplasm	
Control	100	100	100
51°C for 10 min	86	81	10
10 mM EDTA	96	80	34

[a] *E. coli* BB was used to obtain the periplasmic fraction. Heat treatment was performed prior to enzyme analysis. EDTA was present during the enzyme assay. Pure enzyme represents "protease I," kindly provided by M. Pacaud and J. Uriel.

TABLE 4. *Casein-hydrolyzing activity of extracts of Pr^+ and Pr^-*[a]

Strain	Fraction	APNE-hydrolyzing activity		Casein-hydrolyzing activity	
		U/mg of protein	% Total[b]	U/mg of protein	% Total[b]
Pr^+	Stage I shock fluid	–[c]	–	0	0
	Periplasm	8.62	85	3.96	60
	Cytoplasm	0.52	15	0.71	32
	Membrane	–	–	1.02	8
Pr^-	Stage I shock fluid	–	–	0	0
	Periplasm	0	0	3.58	61
	Cytoplasm	0.32	100	0.82	39
	Membrane	–	–	–	–

[a] Strains Pr^+ and Pr^- were grown on Penassay broth and harvested in log phase, and subcellular fractions were prepared as described in the text.

[b] Sum of the activities in all fractions measured.

[c] –, Not tested.

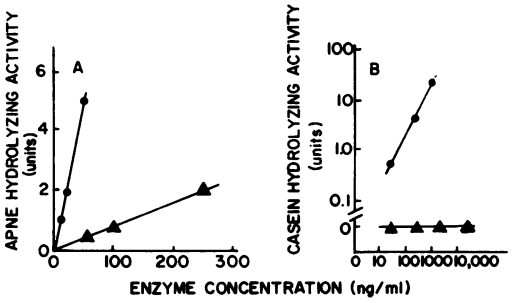

FIG. 1. *Comparison of APNE-hydrolyzing activity (A) and casein-hydrolyzing activity (B) of chymotrypsin (●) and pure APNE-hydrolyzing enzyme (▲).*

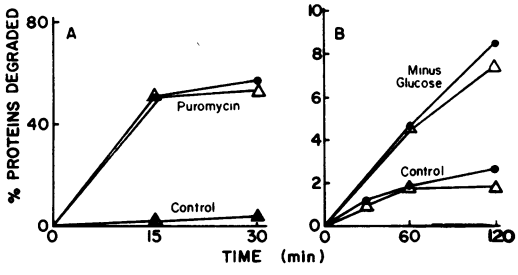

FIG. 2. *(A) Degradation of puromycin-containing and normal proteins by Pr⁺ (●) and Pr⁻ (△). Strains Pr⁺ and Pr⁻ were grown on glycerol minimal medium to log phase. One-half of each culture was incubated with [³H]leucine (0.1 μCi/ml) for 5 min and then washed and suspended in fresh medium containing excess unlabeled leucine. The other half was treated with 200 μg of puromycin per ml for 12 min and subsequently labeled with [³H]leucine (0.1 μCi/ml) for 5 min. Cells were then washed and suspended in fresh medium containing excess unlabeled leucine. After resuspension in fresh medium (zero time), samples taken at various times were mixed with cold 10% trichloroacetic acid and centrifuged, and the acid-soluble radioactivity was determined. (B) Degradation of normal proteins during growth and glucose starvation. Strains Pr⁺ (●) and Pr⁻ (△) were grown on minimal medium with glucose and labeled for two generations with [4,5-³H]leucine (0.1 μCi/ml). Cells were then washed and suspended in fresh medium containing excess unlabeled leucine or in the same medium from which glucose (the only carbon source) had been omitted. Samples were removed at various times after suspension and were mixed with cold trichloroacetic acid. After centrifugation, the acid-soluble radioactivity was determined.*

tion (19). Pr⁻ cells degraded puromycin-containing polypeptides as rapidly as Pr⁺ cells (Fig. 2A).

It is also possible that the APNE-hydrolyzing enzyme may be important in hydrolysis of peptides released from proteins during degradation. Such an activity would have escaped detection in the previous in vivo experiments, which measured only the conversion of acid-insoluble radioactivity to the acid-soluble form. To test this possibility, cells were grown on [*carboxy*-¹⁴C]phenylalanine to label proteins and then transferred to fresh medium with excess unlabeled phenylalanine to follow degradation. Hydrolysis of [¹⁴C]phenylalanine peptides released during protein catabolism leads to the production of new terminal ¹⁴COOH groups. The APNE-hydrolyzing enzyme should cleave specifically on the carboxyl side of phenylalanine residues (17). If the enzyme hydrolyzes such peptides in vivo, its absence should decrease the number of new ¹⁴COOH groups produced during protein degradation. [¹⁴C]-phenylalanine peptides released during protein degradation were hydrolyzed as well by Pr⁻ as by Pr⁺ (Fig. 3). Thus, the APNE-hydrolyzing enzyme, which cleaves the phenylalanine ester APNE, does not appear to be necessary for the cleavage of phenylalanine peptides in protein catabolism. These experiments indicate that the APNE-hydrolyzing enzyme is required for neither the early nor the late steps of protein degradation.

Finally, the APNE-hydrolyzing enzyme may serve to degrade exogenous peptides. Trial experiments showed, in fact, that a phenylalanine-requiring derivative of strain BB (Pr⁺) can utilize the dipeptide phenylalanylphenylalanine for growth. If the APNE-hydrolyzing enzyme is the only, or the major, enzyme capable of degrading this dipeptide, Pr⁻ Phe⁻ cells should not be able to use this dipeptide for growth. However, Pr⁻ Phe⁻ cells and Pr⁺ Phe⁻

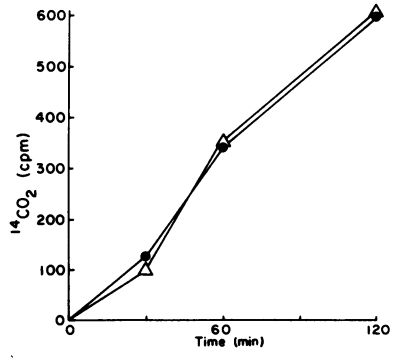

FIG. 3. *Hydrolysis of phenylalanine-containing peptides produced by protein catabolism in Pr⁺ (●) and Pr⁻ (△). Pr⁺ and Pr⁻ were labeled overnight with [1-¹⁴C]phenylalanine and then washed and suspended in fresh medium containing excess unlabeled phenylalanine. At various times, samples were removed, and carboxyl-¹⁴COOH groups were measured as described in the text.*

cells grew at the same rate when supplied with a limiting amount of phenylalanylphenylalanine (Fig. 4). In addition, phenylalanyl tyrosine, phenylalanyl alanine, phenylalanyl-methyl ester, and phenylalanyl-ethyl ester (20 μg/ml) each permitted normal growth rates with both Pr^+ and Pr^- cells. Thus, either the residual cytoplasmic APNE-hydrolyzing activity or some other enzymes are responsible for the utilization of these compounds. This result, of course, does not preclude some minor contribution of the APNE-hydrolyzing enzyme to degradation of such peptides and esters.

DISCUSSION

A number of findings strongly suggest that the enzyme designated "protease I" is not a protease. (i) Subcellular extracts prepared from the Pr^- mutant, which lacks more than 90% of the APNE-hydrolyzing activity, had normal proteolytic activity against casein (Table 4). (ii) The APNE-hydrolyzing activity and proteolytic activity (against casein) from the periplasm had different pH optima and different sensitivities to heat and EDTA (Table 5). (iii) Purified APNE-hydrolyzing enzyme was inactive against denatured collagen and myoglobin (17) as well as casein (Fig. 1) and denatured creatine kinase (unpublished data). The data of Pacaud and Uriel (17), Thang et al. (22), and Hermier et al. (10) suggested that this enzyme is a protease. When these workers incubated protein substrates with the APNE-hydrolyzing enzyme, they observed only limited cleavage of the protein as estimated by sodium dodecyl sulfate-gel electrophoresis. Even this result required unusually long incubation times (7 days at 4°C or 30 h at 20°C) and very high concentrations of the APNE-hydrolyzing enzyme (100 to 600 μg/ml). It is possible that the enzyme preparation had a minor proteolytic contaminant that caused this low amount of protein cleavage. Alternatively, this enzyme, which can clearly function as an esterase, may have a very low hydrolytic activity toward certain proteins. However, until there is stronger evidence for proteolytic activity, we suggest the enzyme would appropriately be called "APNE-hydrolyzing enzyme" or "acetylphenylalanine esterase." The present results illustrate the problems involved in using the hydrolysis of synthetic esters as the sole criterion for defining proteolytic enzymes.

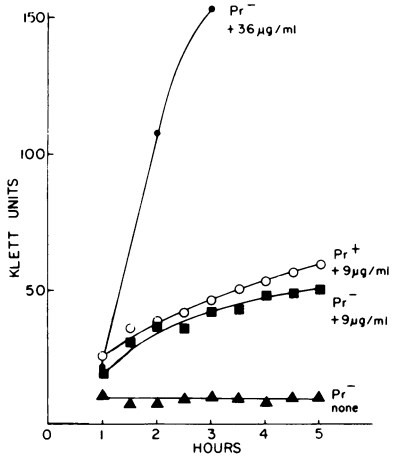

FIG. 4. *Growth of Pr^- Phe^- and Pr^+ Phe^- derivatives of E. coli in minimal medium supplemented with phenylalanylphenylalanine. Overnight cultures, grown in M9 medium supplemented with phenylalanine (40 μg/ml), were washed twice with unsupplemented M9 and finally resuspended in an equal volume of the same unsupplemented medium. At zero time, 0.4 ml of the washed cells was added to 10 ml of M9, with and without the dipeptide, and incubated with aeration at 37°C. The turbidity was followed with a Klett-Summerson colorimeter at 660 nm.*

Experiments with intact cells indicated that the APNE-hydrolyzing enzyme is not essential for intracellular protein degradation. The Pr^- mutant did not differ from the wild type in its ability: (i) to degrade cell proteins during growth; (ii) to degrade cell proteins at increased rates during starvation; (iii) to degrade incomplete polypeptides resulting from incorporation of puromycin; (iv) to hydrolyze phenylalanine-containing peptides released upon degradation of proteins; or (v) to utilize exogenous phenylalanyl peptides or esters for growth.

The APNE-hydrolyzing activity is located primarily in the periplasm, where its specific activity is about 20-fold higher than in the cytoplasm. Pacaud and Uriel (17) found three bands capable of APNE-hydrolysis on acrylamide gel electrophoresis of extracts, one major band which they designated "protease I" and two minor bands whose activity varied from extract to extract. In the Pr^- mutant, only the major activity band is absent (unpublished data). The present results indicate that, in Pr^-, APNE-hydrolyzing activity is undetectable in the periplasmic fraction, whereas a small variable amount of activity is found in the cytoplasm. Thus, cytoplasmic APNE-hydrolyzing activity may represent a second enzyme.

Proteolytic activity was found in all subcellular fractions of *E. coli* (Table 2), in agreement with the observations of Chaloupka (3) and Regnier and Thang (21). The amount of protease released by osmotic shock depended upon

the growth conditions. When cells were grown on Pennassay broth, the osmotic shock released almost 50% of the cells' total proteolytic activity and about 20% of the cell protein. In contrast, when cells were grown on medium B, only 15% of the proteolytic activity and 8% of the protein were released by the same shock procedure. Regnier and Thang (21) also found about 10% of total proteolytic activity in the periplasm when cells were grown on a minimal medium similar to medium B. Chaloupka (3) reported 30% of the proteolytic activity in the periplasm, although the nature of the growth media and degree of cell lysis were not indicated. Perhaps these differences indicate the existence of a second compartment, other than the periplasm, that contains protease and that is released by osmotic shock only from cells grown on a rich medium. Alternatively, these differences may reflect the inducibility of periplasmic proteases by growth on a rich medium.

The specific activity of proteolytic activity in the periplasm is about two- to fourfold higher than that of the cytoplasm (Table 2). A wide variety of degradative enzymes including deoxyribonuclease, ribonuclease, and acid phosphatase are found in the periplasm of gram-negative bacteria (9). Several possible functions have been suggested for these periplasmic hydrolases, including digestion of exogenous substrates unable to pass through the inner cell membrane, digestion of dead cells to make valuable cell constituents available to surviving members of the population, and protection of cells against toxic factors in the environment, such as phage or colicins (9). In addition, periplasmic proteases may function in the degradation of cellular proteins, such as membrane or other periplasmic components. Cytoplasmic proteins may even be transported to the periplasm for degradation. Such a model is analogous to that proposed for the hydrolysis of eukaryotic proteins in lysosomes.

Methods similar to those used here have also been employed to isolate mutants of *E. coli* deficient in a trypsin-like enzyme that hydrolyzes benzoyl-DL-arginine-β-naphthylamide (BANA) (unpublished data). This selection utilized BANA in place of APNE and Fast Garnet GBC Salt in place of Fast Blue Salt B. Our partial purification and characterization of the BANA-hydrolyzing enzyme suggests that it is similar and perhaps identical to "protease II" (16). Mutants (called Tr^-) that lack this hydrolytic activity also show no defect in the degradation of abnormal proteins (e.g., puromycin-containing or analogue-containing polypeptides) or normal proteins during starvation (unpublished data).

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