Biochimica et Biophysica Acta, 445 (1976) 406—419

BBA 67892

PURIFICATION AND PROPERTIES OF A NEW AMINOPEPTIDASE FROM *ESCHERICHIA COLI* K12 *

LIAN-MEI YANG ** and R.L. SOMERVILLE

Department of Biochemistry, Purdue University, West Lafayette, Ind. (U.S.A.)

(Received November 11th, 1975)
(Revised manuscript received April 27th, 1976)

Summary

An aminopeptidase (EC 3.4.11.-) capable of hydrolyzing L-alanyl-β-naphthylamide and certain other aminoacyl β-naphthylamides was purified to homogeneity from extracts of *Escherichia coli* K-12. The enzyme, designated aminopeptidase II, is a monomeric protein of mol. wt. 100 000. It exhibits a broad pH optimum in the range pH 7.0—9.0. Although Zn^{2+}, Fe^{3+} and Cr^{3+} are strong inhibitors of enzyme activity, a metal requirement for catalysis could not be firmly established. Neither sulfhydryl reagents nor serine protease inhibitors affected enzyme activity.

The nature and physiological roles of intracellular enzymes functional in the hydrolysis of peptide bonds in bacteria has attracted increased attention in recent years [1,2]. Several authors have considered the possible roles of peptidases in peptide utilization and transport [3], enzyme maturation [4], protein synthesis [5], recognition and degradation of protein fragments and incorrectly folded intracellular proteins [6] and the breakdown of correctly assembled proteins during normal growth or following a shift in physiological conditions [7].

Attempts to assign specific roles in one or more of the above processes to a given peptidase have been complicated by the fact that many of the enzymes studied in cell-free systems have broad, overlapping specificities with respect to the bonds cleaved. Thus a loss through mutation of one enzyme might be readily tolerated by a cell because of the presence of other peptidases capable of supplying the missing function.

We elected to approach the problem in a systematic manner by first studying

Abbreviation: SDS, sodium dodecyl sulfate

* Journal Paper No. 6078 of the Purdue Agricultural Experiment Station.

** Present Address: Department of Pharmacology, University of Wisconsin, Madison, Wisc. (U.S.A.)

an *Escherichia coli* enzyme of known specificity which could be readily assayed in vitro and in intact cells. Our rationale was to characterize one such enzyme through purification, then attempt to isolate mutants defective in that protein so that its role in a given cellular process might be ascertained.

After the completion of our work, we learned that Miller et al. applied a similar line of reasoning in genetic studies of proteolysis in *Salmonella typhimurium* [8,9]. Our results complement their findings. They have isolated mutants (*pepN*) presumably unable to form the enzyme (aminopeptidase II) (EC 3.4.11.-). we have purified. Physiological studies with mutants incapable of forming the *pepN* gene product suggest that aminopeptidase II may be important in carbon-starvation protein turnover, which in turn may be related to cellular requirements for new protein following a shift-down from rich to poor medium [10].

Methods

Materials

Aminoacyl-β-naphthylamides and peptidyl-β-naphthylamides * were purchased from the Sigma Chemical Company, ICN Pharmaceuticals, Schwarz/Mann Division, Becton-Dickinson and Company, or Fox Chemical Company. Fast Garnet GBC was obtained from the Sigma Chemical Company. Phenylmethanesulfonyl fluoride was purchased from Calbiochem.

Sephadex G-200 and DEAE-Sephadex A50 were obtained from Pharmacia Fine Chemicals and used as directed in the Pharmacia manual. Hydroxyapatite and the chemical ingredients used in polyacrylamide disc gel electrophoresis were purchased from Bio-Rad Laboratories. Diaflo membranes were obtained from the Amicon Corporation. Dialysis tubing was a product of Union Carbide.

Crude extracts were prepared by sonication from frozen cell paste of *E. coli* K-12 which had been cultured in casein hydrolysate media to full growth (Grain Processing Corporation, Muscatine, Iowa). Trypsin, trypsin inhibitors (soybean, lima bean and pancreatic) and crystalline yeast alcohol dehydrogenase were obtained from the Worthington Biochemical Corporation. Crystalline bovine liver glutamic dehydrogenase was obtained from Calbiochem. Bovine serum albumin was purchased from Miles Laboratories.

Enzyme assays

The continuous enzymatic hydrolysis of L-alanyl-β-naphthylamide and other aminoacyl-β-naphthylamides was monitored fluorimetrically (Fig. 1).

Standard assay mixtures contained, in a total volume of 1 ml, 0.05 M Tris buffer at pH 7.5, 1 mM L-alanyl-β-naphthylamide and enzyme, which was added last to initiate the reaction **. The release of free β-naphthylamine was fol-

* Unless otherwise specified, amino acids and aminoacyl derivatives used in this work were all of the L configuration.

** After most of the experiments described herein had been completed, we discovered that inclusion of bovine serum albumin at levels up to 2.5 mg per assay resulted in enhancements of up to 50% in the rate of hydrolysis of alanyl-β-naphthylamide. The stimulatory effect was not a result of increases in the fluorescence yield of β-naphthylamine. From Lineweaver-Burk plots it appears that bovine serum albumin leads to increases in V without affecting K_m. The basis of this observation is at present not clear.

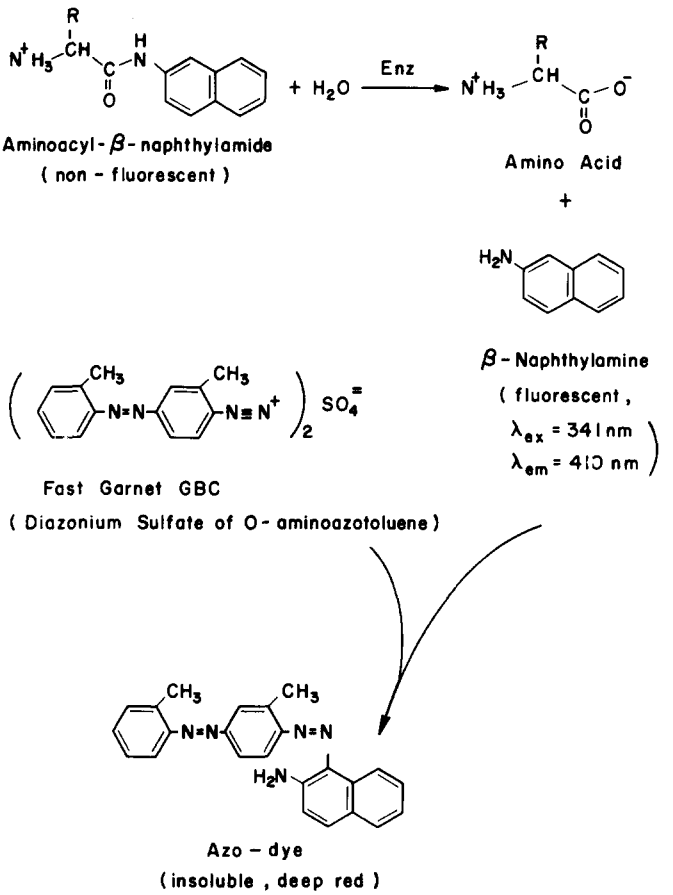

Fig. 1. General reaction scheme for measurement of hydrolysis of aminoacyl-β-naphthylamides. Direct observation of β-naphthylamine liberation utilizes fluorometric procedures. For activity stains in whole cells or following the electrophoretic separation of proteins on gels one employs the stable diazonium salt Fast Garnet GBC. (See text).

lowed in an Aminco-Bowman spectrophotofluorometer. Fluorescence was recorded continuously on a Sargent SR time-base recorder. The excitation and emission wavelengths were 341 nm and 410 nm, respectively. One unit of enzyme is that amount which releases 1 mmol of β-naphthylamine per min at 25°C. Specific activity is enzyme units per mg protein. The initial rate of hydrolysis of alanyl-β-naphthylamide was proportional to the amount of enzyme up to at least 1.1 μg of pure enzyme in 1 ml of standard assay mixture. Given the sensitivity of the fluorescence detection system for free β-naphthylamine this proportionality should hold below 0.1 μg/ml of enzyme.

Protein concentrations were determined according to the method of Lowry et al. [11] with crystalline bovine serum albumin as standard.

Electrophoresis of protein solutions was conducted in 5% polyacrylamide gel using Tris/glycine buffer, pH 8.3 [12]. The samples usually contained 0.02—0.1 mg protein. A voltage gradient sufficient to produce a current flow of 2 mA per gel was applied. After each gel was removed from its tube, protein bands were

visualized by staining in Coomassie brilliant blue, prepared according to Weber and Osborn [13] for 30 min. Gels were destained overnight in a mixture of ethanol, acetic acid and water in the ratio of 6 : 1 : 13.

To detect enzyme activity (Fig. 1) gels were immersed in a small volume of enzyme assay mixture for 15—30 minutes, depending on the stage of purification. Staining was achieved following immersion for a further 30 min in a solution of Fast Garnet GBC prepared according to the method of Marks et al. [14] (i.e. 0.14% Fast Garnet GBC in 0.2 mM acetate, pH 4.5).

Sodium dodecyl sulfate (SDS) gels were prepared according to the method of Fairbanks et al. [15]. Solutions containing protein at a concentration of 0.01 mg/ml were dialyzed against 10 mM Tris · HCl, 1 mM EDTA pH 8 and 1% SDS overnight at room temperature. The dialyzed material was incubated with dithiothreitol (20 mM) for 30 min, then layered onto a gel. Electrophoresis was conducted at 5 mA per gel. The protein was stained with Coomassie brilliant blue for 2 h, then destained overnight as before.

Results

Activity in crude extracts

Eleven aminoacyl-β-naphthylamides were tested for hydrolysis by enzymes present in crude extracts of *E. coli* K-12 (Table I). Alanyl-β-naphthylamide was the most susceptible substrate. Lysyl-β-naphthylamide and arginyl-β-naphthylamide were hydrolyzed at 15—20% of the rate of alanyl-β-naphthylamide. The remainder were hydrolyzed at a rate of less than 5% of that observed for alanyl-β-naphthylamide (Columns 1 and 2, Table I). An activity stain following polyacrylamide gel electrophoresis of crude extracts showed only one deep red band (Fig. 2). This suggested that *E. coli* K-12 contains only one enzyme active toward alanyl-β-naphthylamide. For this reason we focused attention on the enzymatic hydrolysis of alanyl-β-naphthylamide. Later we established that a single enzyme hydrolyzed alanyl-β-naphthylamide, arginyl-β-naphthylamide and lysyl-β-naphthylamide (Columns 3 and 4, Table I).

Purification of aminopeptidase II

A typical purification scheme is given in the legends to Table II and Fig. 3. From 40 g of wet cells we obtained 4.76 mg of homogeneous enzyme. This represented about 8.5% of the material originally present in the crude extract.

State of purity

The enzyme as eluted from DEAE showed a single band upon polyacrylamide gel electrophoresis and staining either for protein or enzyme activity. Only one band was detectable on SDS gel (Fig. 2). Even though the mobility of our enzyme on SDS gel is less than that of the reference proteins, it is valid to estimate molecular weight by extrapolation [13].

Dependence of enzyme activity on hydrogen ion concentration

The enzyme has a broad pH optimum between pH 7.0 and 9.0, with a maximum at approximately pH 8.5 (Fig. 4). At a given pH, the activity measured in different buffers showed slight but significant variations. This variability may

TABLE I

HYDROLYSIS OF VARIOUS L-AMINOACYL-β-NAPHTHYLAMIDES

The crude extract and purified enzyme belonged to the same preparation. The purified enzyme was a DEAE-Sephadex fraction which was prepared according to established purification procedures. It had only one faint contaminating band on gel electrophoresis and was estimated visually to be 90% pure. When freshly prepared, it had a protein concentration of 1.1 mg/ml, and a specific activity of 20. The hydrolysis of alanyl-β-naphthylamide and lysyl-β-naphthylamide were measured during the initial one or two minutes after adding enzyme; the small amount of hydrolysis of the other amino acyl-β-naphthylamides were obtained on incubation for a longer time, and the rates were calculated from the linear portion of the hydrolysis curve.

Aminoacyl-β-naphthylamide (aminoacyl moiety)	Crude extract *		Purified enzyme **	
	Activity units $\times 10^{-3}$	Relative to Ala (%)	Activity units $\times 10^{-3}$	Relative to Ala (%)
L-Ala	10.03	100	4.02	100
***L-Arg	1.80	18.0	0.82	20.4
***L-Lys	1.56	15.6	0.72	17.9
L-Ser	0.33	3.3	0.19	4.6
L-Leu	0.14	1.4	0.11	2.8
L-Met	0.10	1.0	0.07	1.8
L-Pro	0.07	0.7	0.04	0.9
L-Thr	0.05	0.5	0.03	0.6
L-His	0.05	0.5	0.02	0.5
L-Val	0.04	0.4	0.02	0.6
L-Ile	0.01	0.1	0.00	0.0
L-Asp	0.00	0.0	0.00	0.0
L-Ala			0.00	0.0

* Another preparation of crude extract gave similar results.

** A second experiment using the same purified enzyme gave similar results, except that the rates of hydrolysis of leucyl-β-naphthylamide and methionyl-β-naphthylamide were 2.0 and 1.6% respectively, of the rate of alanyl-β-naphthylamide hydrolysis.

*** A separate preparation of purified enzyme gave similar percentage of hydrolysis of lysyl-β-napthylamide and arginyl-β-naphthylamide relative to alanyl-β-naphthylamide.

be attributable to ionic strength differences, for which no corrections were made.

Molecular weight

The molecular weight of aminopeptidase II, estimated from its degree of retention by Sephadex G-200, was 100 000. Following gel electrophoresis in sodium dodecyl sulfate, we observed a single protein band corresponding to a monomer of molecular weight 108 000 (Fig. 5). These data suggest that the enzyme consists of a single polypeptide chain.

Divalent metal ion studies

Overnight dialysis of aminopeptidase II against 0.1 M Tris buffer, pH 7.8, containing 2 mM EDTA led to only partial loss of activity. No significant stimulation of activity was observed upon adding a series of metal ions at various concentrations to the dialyzed enzyme (Table III). Some metal ions, such as Fe^{3+}, Cr^{3+}, Zn^{2+} and Hg^{2+}, were strongly inhibitory.

In a separate series of experiments, the metal chelating agents 8-quinolinol,

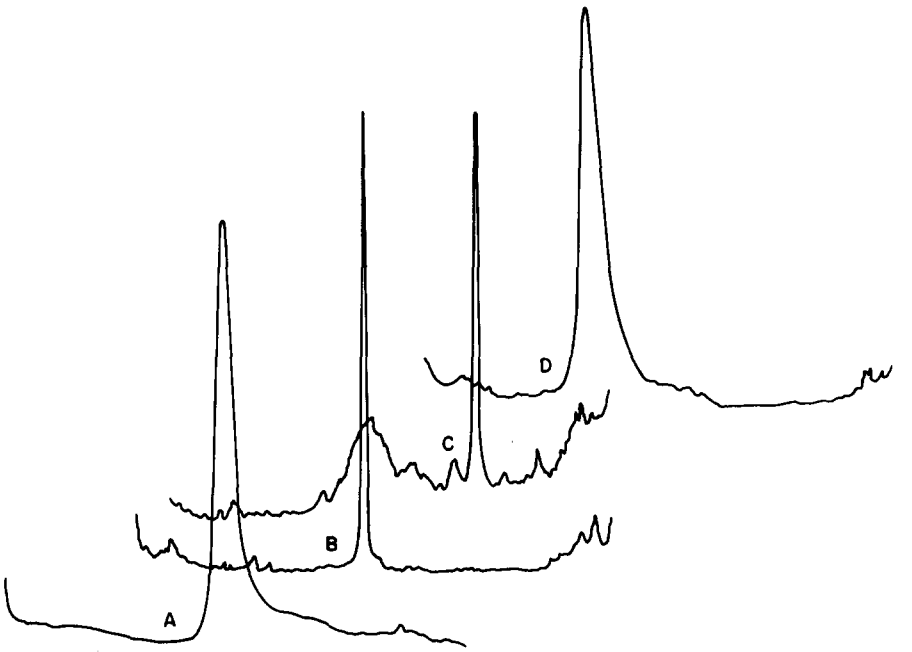

Fig. 2. Densitometer tracings of stained polyacrylamide gels. Gels prepared as described in the text were scanned at 550 nm in a Gilford spectrophotometer using the appropriate attachments. A. Purified enzyme (18 μg) (cf. Table II) stained for enzyme activity. Peak absorbance = 1.5. B. Purified enzyme (18 μg) stained for protein. Peak absorbance = 3.0. C. Purified enzyme (10 μg) stained for protein following denaturation and electrophoresis on SDS gel (see text). Peak absorbance = 2.8. D. Crude extract (160 μg) stained for enzyme activity. Peak absorbance = 1.4. The direction of migration was from left to right.

α,α′-dipyridyl, *o*-phenanthroline and 8-hydroxy-5-quinolinesulfonate were tested with respect to their ability to inhibit the enzymatic hydrolysis of alanyl-β-naphthylamide by purified enzyme under standard assay conditions. When the chelators were present at concentrations of 10^{-4} M, little if any inhibition (4—10%) was noted. At higher concentrations (10^{-3} M) greater inhibition (15—93%) became evident. The chelator *o*-phenanthroline was the most effective inhibitor, but attempts to restore activity by adding divalent metal ions to enzyme preparations were only marginally successful. For example, upon incubation of an *o*-phenanthroline-inhibited sample with 10^{-3} M Mn^{2+}, an increase in activity from 14% of control values to 37.6% of control rates was observed. In contrast, 10^{-3} M Mn^{2+}, when added to enzyme solutions which had been 60—70% inhibited by 10^{-3} M 8-quinolinol or 8-hydroxy-5-quinolinesulfonate, produced further inhibition.

Effects of sulfhydryl reagents

Aminopeptidase II proved to be relatively resistant to inactivation or inhibition by sulfhydryl reagents. In enzyme assay mixtures, inhibition by iodoacetamide, *N*-ethylmaleimide and *p*-chloromercuribenzoate became significant only when the inhibitor concentrations exceeded 5 mM. The addition of an equimolar quantity of 2-mercaptoethanol to enzyme preparations which had been incubated with *N*-ethylmaleimide (5 mM) or *p*-chloromercuribenzoate (5 mM) for 10 min at 25°C restored most of the lost activity. In a separate experi-

TABLE II

PURIFICATION OF *E. COLI* AMINOPEPTIDASE II

40 g of *E. coli* K-12 cell paste was suspended in 200 ml 0.1 M Tris buffer, pH 7.8. The suspension was sonicated for 45 min with a Branson W185C Sonifier (power setting, 5). During extraction the cells were maintained at or below 10°C in a salt/ice bath. All subsequent treatments were done at 0—4°C. The cell debris was removed by centrifugation in a Sorval RC-2B centrifuge at 48 000 × *g* for 40 min. The crude extract was diluted to approximately 15 mg/ml protein by adding 155 ml Tris buffer (0.1 M, pH 7.8). Then 19 ml of 1.0 M $MnCl_2$ was added drop by drop, with constant stirring. After the suspension had been stirred for an additional 20 min, the precipitate was removed by centrifugation at 48 000 × *g* for 15 min. The supernatant was further fractionated by adding solid ammonium sulfate to isolate proteins precipitating between 42.5% and 57.4% saturation. The protein pellet was dissolved in 0.1 M Tris buffer, pH 7.8. Further purification involved fractionation by passage through Sephadex G-200 columns (2.5 × 85 cm) preequilibrated and eluted with 0.1 M Tris, pH 7.8. To prevent overloading, 400 mg protein or less was applied during any given run. Fractions from each column with high specific activities were combined and concentrated by ultrafiltration under N_2 pressure in a Diaflo apparatus using a PM 30 membrane. The concentrated material (11 ml) from four Sephadex G-200 columns was dialyzed overnight against 2 l of 0.025 M potassium phosphate buffer, pH 6.8. A slight precipitate which formed during dialysis was removed by centrifugation at 20 000 rev./min for 15 min. The supernatant was layered on a hydroxyapatite column (2.5 × 26 cm) preequilibrated with 0.025 M phosphate buffer at pH 6.8. The column was eluted with a linear gradient of phosphate buffer at pH 6.8. The phosphate concentration rose from 0.025 M to 0.175 M during the passage of 800 ml of solution. The most active enzyme fractions were pooled and concentrated to 9 ml by ultrafiltration. Purification to homogeneity was accomplished by chromatography on DEAE-Sephadex A-50 (Fig. 3).

Step	Volume (ml)	Protein conc. (mg/ml)	Specific activity (units/mg protein)	Purification (fold)	Yield * (%)
Crude extract	227	26.4	0.16	1.0	
$MnCl_2/(NH_4)_2SO_4$	48	30.0	0.24	1.5	(100)
Sephadex G-200	11	24.2	0.90	5.6	73.7
Hydroxyapatite	9	5.0	4.70	29.4	68.1
DEAE-Sephadex	6.8	0.7	17.16	107.3	26.6

ment, 9.58 munits of enzyme were incubated in assay buffer with freshly prepared *N*-ethylmaleimide (5 mM). During a 20-min incubation period at 25°C, samples were withdrawn, diluted 1000-fold in assay buffer and assayed for activity by the standard procedure. More than 90% of the original activity remained after exposure to *N*-ethylmaleimide. These results tend to suggest that no highly reactive sulfhydryl groups are mechanistically connected to the active center of aminopeptidase II.

Effects of monovalent ions

Vogt [16] and Mäkinen and Mäkinen [17] earlier showed that NaCl and KCl interacted with aminopeptidases from *E. coli* and rat liver. Depending on their experimental conditions, either activation or inhibition could be demonstrated. No such interactions take place with our enzyme. When NaCl or KCl (0.05—1.0 M) was included in reaction mixtures the observed hydrolysis rates deviated by no more than 10% from the rates of control reactions.

Serine protease inhibitors

Soybean, lima bean and pancreatic trypsin inhibitors failed to inhibit catalysis when they were included in assay mixtures at levels 8—40-fold higher than

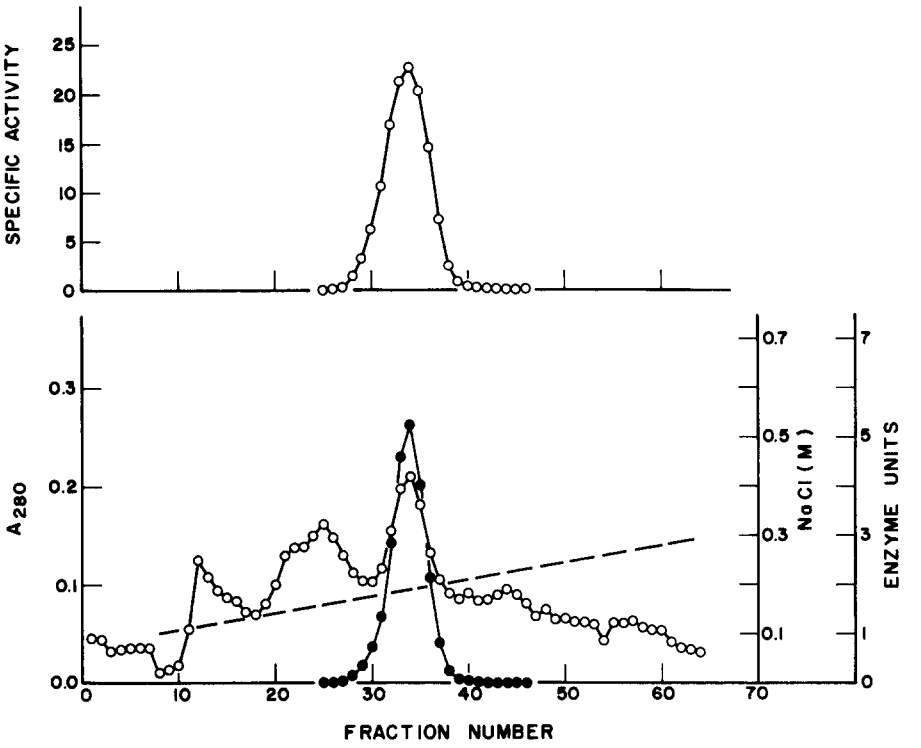

Fig. 3. Final purification step in the preparation of *E. coli* aminopeptidase II. A DEAE-Sephadex A-50 column (1.74 × 34 cm) was pre-equilibrated with 0.1 M potassium phosphate buffer, pH 6.8. Following the addition of dialyzed, concentrated hydroxyapatite fraction (see legend to Table II), elution was conducted at a flow rate of 8 ml/h. A linear gradient of NaCl (0.1—0.3 M) was applied during the passage of 400 ml of eluant. Fractions of approximately 6 ml volume were collected. (○) A_{280} and specific activity; ●, Aminopeptidase II activity; - - - - - -, NaCl concentration. The most active fractions were pooled and concentrated by ultrafiltration.

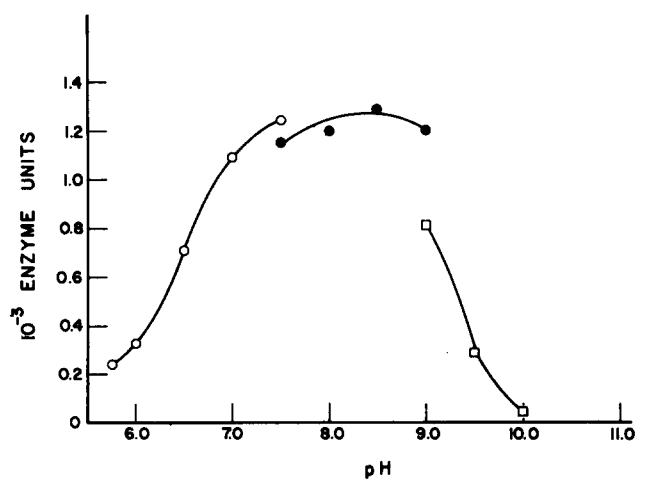

Fig. 4. Variation in rate of alanyl-β-naphthylamide hydrolysis with respect to hydrogen ion concentration. The purified enzyme fraction was the same one used in the experiment of Fig. 7. Standard assays were conducted in the following buffers: ○, potassium phosphate; ●, Tris; □, sodium bicarbonate.

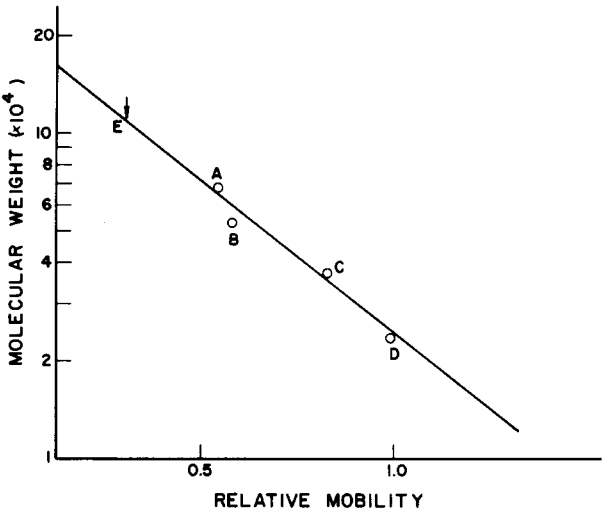

Fig. 5. Molecular weight estimation for purified aminopeptidase II. See text for a description of the procedure used. The standards employed were: A, bovine serum albumin; B, glutamate dehydrogenase (bovine liver); C, alcohol dehydrogenase (yeast); D, Trypsin; E refers to the mobility of purified, denatured aminopeptidase II.

that of enzyme (weight). Phenylmethanesulfonylfluoride at concentrations up to 1 mM also showed no effect.

Kinetic constants; substrate inhibition

From double reciprocal plots the K_m (apparent) with respect to alanyl-β-naphthylamide for pure enzyme was $1.85 \cdot 10^{-4}$ M; with 0.11 μg ($1.1 \cdot 10^{-6}$ μmol) of pure enzyme the V was $4.65 \cdot 10^{-3}$ enzyme units. The turnover number is therefore $4 \cdot 10^{6}$. However, the actual maximal attainable activity is only

TABLE III

EFFECTS OF METAL IONS ON THE ACTIVITY OF DIALYZED AMINOPEPTIDASE II

All the metals were present as the chloride salts. The purified enzyme (see legend to Table II) was dialyzed against Tris · HCl buffer, 0.1 M, pH 7.8, containing 2 mM EDTA overnight. Various metals at the indicated concentrations were added to the assay mixture at the start of the reaction to ascertain their effect on the activity of dialyzed enzyme. The data shown are munits of enzyme activity.

Cation	Cation conc.			
	$5 \cdot 10^{-4}$ M	$5 \cdot 10^{-5}$ M	$5 \cdot 10^{-6}$ M	O
Ca^{2+}	0.80	1.00	0.98	
Mg^{2+}	0.65	1.00	1.02	
Mn^{2+}	0.63	0.73	1.09	
Co^{2+}	0.51	0.65	0.77	
Ni^{2+}	0.39	0.59	0.74	
Cu^{2+}	0.20	0.56	1.01	
Zn^{2+}	0.03	0.18	0.86	
Hg^{2+}	0.04	0.47	0.93	
Cr^{3+}	0.02	0.05	0.39	
Fe^{3+}	0.06	0.24	0.40	
Control A (before dialysis)				2.50
Control B (after dialysis)				0.86

$2 \cdot 10^6$ mol of product per minute per mol of enzyme, because substrate inhibition sets in when the concentration of alanyl-β-naphthylamide exceeds 1 mM.

Substrate specificity

The comparative ability of a set of aminoacyl-β-naphthylamides to serve as substrates for purified aminopeptidase II is documented in columns 3 and 4, Table I. Alanyl, lysyl, and arginyl-β-naphthylamides were hydrolysed most effectively. The relative rates for all substrates with pure enzyme were very similar to those observed with crude extract. This suggests that *E. coli* contains a single enzyme possessing hydrolytic activities towards numerous aminoacyl-β-naphthylamides.

When aminoacyl-β-naphthylamides which were relatively inert as substrates were present in assay mixtures, the hydrolysis of alanyl-β-naphthylamide was inhibited to varying degrees (Table IV). Therefore non-hydrolyzable aminoacyl-β-naphthylamides are capable of binding to the enzyme. This was explored in a series of kinetic experiments. A Dixon plot of inhibition by leucyl-β-napthylamide showed intersecting straight lines. This suggested that this compound binds competitively with alanyl-β-naphthylamide at a substrate site. In contrast, a Dixon plot where valyl-β-naphthylamide was treated as an inhibitor gave a pattern of nonintersecting lines showing clear-cut upward curvatures.

Arginyl- and lysyl-β-naphthylamides, furnished at saturating concentrations, were hydrolyzed slowly (Table I). Somewhat surprisingly, the apparent K_m values for both of these substrates (approx. $5 \cdot 10^{-7}$ M) was more than three orders of magnitude less than that determined for alanyl-β-naphthylamide. In the absence of additional information we are unable to state whether this large difference is related to binding, catalysis, or both.

The purified enzyme did not hydrolyze D-alanyl-β-naphthylamide (Table I).

TABLE IV

INHIBITION OF AMINOPEPTIDASE II BY NON-HYDROLYZABLE AMINOACYL-β-NAPHTHYLAMIDES

The results shown were obtained in two separate experiments using purified enzyme (Table I). All aminoacyl-β-naphthylamides were present at concentrations of 1 mM (Exp. 1) or 0.5 mM (Exp. 2). The substrate in every case was alanyl-β-naphthylamide, present at the same concentration as the inhibitor.

Aminoacyl-β-naphthylamide added to standard assay	Exp. 1		Exp. 2	
	Activity (units × 10^{-3})	% of control	Activity (units × 10^{-3})	% of control
None	2.96	100	8.37	100
Gly	—	—	6.23	74.5
Val	0.96	32.5	3.01	35.9
Leu	0.29	9.6	1.27	15.2
Ile	0.49	16.6	2.39	28.5
Ser	2.10	70.9	5.15	61.5
Thr	2.08	70.3	5.95	71.2
Asp	1.90	64.1	5.25	62.8
His	1.56	52.7	2.96	35.4
Met	0.46	15.6	2.07	24.8
Pro	2.14	72.3	5.33	63.8
D-Ala	—	—	6.02	71.9

Nor was the hydrolysis of L-alanyl-β-naphthylamide inhibited by the D isomer, so the D isomer probably does not bind to the enzyme. These results suggest that the binding site of the enzyme for the aminoacyl moiety is stereospecific for the L configuration of the amino acid.

Glycylglycyl-, lysylalanyl- and alanylalanyl-β-naphthylamides were also tested as substrates for this enzyme. Only alanylalanyl-β-naphthylamide was hydrolyzed to any appreciable extent. When the hydrolysis of alanylalanyl-β-naphthylamide in the presence of suitable levels of enzyme was observed for an extended period of time, a steadily increasing rate of accumulation of β-naphthylamine was observed. Since this tripeptide analog was cleaved, it can be tentatively concluded that this enzyme is not specifically a dipeptidase.

When glycylglycyl- and lysylalanyl-β-naphthylamides were tested for inhibition of alanyl-β-naphthylamide hydrolysis, the former scarcely affected the activity (about 90% remained); the latter showed strong inhibition (85—90%). This suggests that glycylglycyl-β-naphthylamide cannot be hydrolyzed because it fails to bind to the enzyme, and that lysylalanyl-β-naphthylamide can bind to the enzyme, but is not susceptible to hydrolysis.

Inhibitory factor in crude extracts

When the 42.5—57.4% ammonium sulfate fraction was passed through a Sephadex G-200 column, an inhibitory factor was separated from the enzyme. By pooling separate fractions, preparations were obtained which were suitable for preliminary studies of the phenomenon. With increasing amounts of pooled, concentrated inhibitor, progressively more complete inhibition was noted. The inhibition started to level off when approximately 40% of the starting activity remained.

The inhibitory activity, whose mobility was markedly retarded during passage over Sephadex G-200, survived treatment in a boiling water bath for 10 min or exposure to mild acidic conditions (pH 2). The inhibitor readily passed through Diaflo ultrafiltration membrane UM 02, which retains substances with molecular weights greater than 1000. Therefore, the inhibitory material is probably of low molecular weight rather than a protein.

The Sephadex G-200 fractions enriched with respect to inhibitor contained a number of compounds as revealed by paper electrophoresis and paper chromatography. A number of unidentified substances were present, including ninhydrin-reactive, ultraviolet-absorbing and fluorescent materials. At present we are unable to state whether one or more than one compound is responsible for the inhibition.

A possibly related observation is our finding that crude extracts and ammonium sulfate fractions can be activated 2—3 fold by incubation in the presence of 1 mM Co^{2+}. No such effect was noted for enzyme preparations which had been fractionated on Sephadex G-200. We postulate that Co^{2+} stimulation reflects the presence in early fractions of inhibitory substance or substances whose effect can be overcome by interaction with the divalent metal ion. A more detailed understanding of the relationship between Co^{2+} and naturally occurring inhibitors will require additional experimentation.

Physiological variation in measurable enzyme levels during growth

The activity of aminopeptidase II seems to vary during cell growth. The spe-

TABLE V

VARIATION IN AMINOPEPTIDASE II DURING GROWTH OF CELLS IN DIFFERENT MEDIA

Cells of *E. coli* K-12, W3110 were grown at 37°C with vigorous aeration on a rotary shaker. Cells were harvested at cell densities roughly corresponding to $5 \cdot 10^7$/ml, $2 \cdot 10^8$/ml or 10^9/ml, extracted by sonication and assayed.

	Specific activity	
	Minimal + glucose	L-broth
Early log phase	—	0.08
Mid log phase	0.21	0.23
Stationary phase	0.95	1.34

cific activity in the crude extract is higher for stationary-phase cells than for exponentially growing cells (Table V). On the other hand, the effect, if any, of growth media, is only slight. Because of possible complications in assay which might be ascribed to the presence of inhibitors, these results must be regarded as preliminary.

Discussion

The relatively high turnover number of aminopeptidase II with respect to alanyl-β-naphthylamide ($4 \cdot 10^6$) suggests that alanine-containing peptides are important normal substrates. Lysyl- and arginyl-β-naphthylamides were also hydrolyzed with ease, although at a lesser rate. Give the apparent K_m values for alanyl-β-naphthylamide ($4.65 \cdot 10^{-4}$ M) and lysyl- and arginyl-β-naphthylamide ($5 \cdot 10^{-7}$ M), and the structural dissimilarities between lysine and arginine on one hand, and alanine on the other, one must consider the possibility that aminopeptidase II has separate binding sites for these substrates, one specific for alanyl moieties, the other for lysyl and arginyl moieties.

Other lines of evidence support the notion that *E. coli* aminopeptidase II has more than one substrate binding site. The observed substrate inhibition is reminiscent of a classical case studied by Lumry et al. [18], i.e. the carboxypeptidase-catalyzed hydrolysis of carbobenzoxylglycyl-L-tryptophan. The simplest mechanism that explains this type of behavior involves the formation, subsequent to the ordinary Michaelis complex, of a second complex which contains two molecules of alanyl-β-naphthylamide attached to one of enzyme. Since the rate approaches a limiting value lower than the maximum value one would predict that the $E \cdot S_2$ complex reacts less rapidly than the $E \cdot S$ complex.

The compounds L-leucyl-β-naphthylamide and L-valyl-β-naphthylamide are essentially inert as substrates, yet function as effective inhibitors of the hydrolysis of L-alanyl-β-naphthylamide (Table I). The branched chain aminoacyl-β-naphthylamides differ with respect to mode of inhibition. Simple competitive inhibition was noted for L-leucyl-β-naphthylamide whereas inhibition by L-valyl-β-naphthylamide was non-competitive and allosteric in nature. This suggests that distinct binding sites for these inhibitors exist on the surface of aminopeptidase II. An adequate description of the interrelationships between

the various substrate and inhibitor sites on this enzyme will require much additional work.

The fact that aminopeptidase II hydrolyzes alanylalanyl-β-naphthylamide suggests that this enzyme is not specifically a dipeptidase. It might be either an aminopeptidase or an endopeptidase. However, aminoacyl-β-naphthylamides have been considered by other workers to be substrates for aminopeptidases [19]. N-substituted aminoacyl-β-naphthylamides have in the past been found to be substrates for endopeptidases or proteases [20,21]. Therefore, the idea of this enzyme being an aminopeptidase is favored, hence its designation as aminopeptidase II.

Aminopeptidase II was essentially pure after a purification of about 100-fold had been achieved. This enzyme thus constitutes almost 1% of the extractable cellular protein. This relatively high cellular content of aminopeptidase II suggests that it plays an important role.

The apparent specific activity of aminopeptidase II increases as *E. coli* cells enter stationary phase. In this connection, it is interesting to note that stationary cells exhibit higher protein turnover than log phase cells [22]. However, the molecular basis of this variation is unclear. Physiological modulation of the concentrations of natural inhibitor or inhibitors of aminopeptidase II could be one way to control its activity.

Our enzyme differs from all other *E. coli* proteolytic enzymes because of its distinct molecular weight and high intracellular concentration. β-aspartyl peptidase [23] and dipeptidase M [5] bear some superficial similarity to our enzyme. When 2.5 units/ml of purified aminopeptidase II was incubated with 20 mM β-aspartyl-leucine under our standard assay conditions for 2 h, and a sample of the reaction mixture was analyzed by thin-layer chromatography, the starting substrate was completely unchanged. The same experiment done with crude extract (0.8 units/ml), produced complete hydrolysis of β-aspartylleucine. Therefore, it can be concluded that our enzyme is different from β-aspartylpeptidase. Methionylalanine (10 mM) can be completely hydrolyzed by high levels of our enzyme (2.5 units/ml) after 4 h. However, 1 mM methionylalanine inhibited alanyl-β-naphthylamide hydrolysis less than 30%. The lack of significant competition by methionylalanine under our standard assay conditions suggests that the hydrolysis of methionylalanine, although detectable, is not a major activity of our enzyme.

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