STUDIES ON THE SUBSTRATE SPECIFICITY OF AN *ESCHERICHIA COLI* PEPTIDASE

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SUMMARY

The initial rates of hydrolysis of 32 small peptides catalyzed by an enzyme prepared from *Escherichia coli* are reported. The dipeptides tested as substrates were of the following general types: methionyl-X, alanyl-X, glycyl-X and X-alanine where X represents any one of several amino acids. The other compounds examined were tri-, tetra-, and pentapeptides which contained N-terminal methionine. Methionylglycine, methionylalanine, methionylserine and methionylmethionine were more rapidly cleaved than any of the other substrates tested. A possible role for this peptidase in the removal of N-terminal methionine from certain proteins after initiation by N-formylmethionyl-tRNA is discussed.

INTRODUCTION

Synthesis of proteins in *Escherichia coli* is initiated by a process requiring N-formylmethionyl-tRNA (1-3). After initiation, the N-terminal region of the polypeptides is modified (3) to produce the distribution of N-terminal amino acids observed for the native proteins (5). The modification process probably involves at least two distinct steps: the removal of the formyl group and the subsequent hydrolysis of methionine from certain of the newly initiated polypeptides. Enzymes which appear to catalyze the hydrolysis of formate from the nascent chains have been described (5,6,7). Few details concerning the subsequent hydrolysis of methionine are available. Peptidases which promote the cleavage of N-terminal amino acids from small peptides have been reported (6-10) but thus far it does not appear that any of these enzymes are specific for the removal of N-terminal methionine. A study of the substrate specificity of a peptidase which catalyzes the preferential hydrolysis of certain methionyl dipeptides is the subject of this paper.

MATERIALS AND METHODS

All substrates used in these experiments except N-formylmethionylalanine and the pentapeptide Met-Ala-Ser-Asn-Phe were purchased from Mann Research

Corporation or International Chemical and Nuclear Corporation. The pentapeptide was a gift from Dr. John M. Stewart of the Department of Biochemistry, University of Colorado School of Medicine. Methionylalanine was converted to its formyl derivative by the method of Sheehan and Yang (11) and was purified by passage over a Dowex-50 column (12). Escherichia coli B which had been harvested in late log phase and frozen were purchased from General Biochemicals (catalogue number 150020).

The partially purified enzyme used in these studies was prepared at 0-4° as follows. Aliquots of the frozen E. coli B were suspensed in 0.05 M potassium phosphate buffer (pH 7.0) which contained 1 mM β-mercaptoethanol. After thawing, the cells were disrupted by sonic oscillation and the unbroken cells and cell debris sedimented by centrifugation at 15,000 x g for 10 min. The nucleic acids were removed from the resulting supernatant fraction by precipitation with streptomycin sulfate (final concentration 2.5%) and centrifugation at 30,000 x g for 30 min. This solution was dialyzed overnight against 0.01 M Tris-HCl buffer (pH 7.4) containing 1 mM β-mercaptoethanol (TM buffer). The proteins were then fractionated by $(NH_4)_2SO_4$ precipitation. Peptidase activity was found in all $(NH_4)_2SO_4$ precipitated fractions examined; however, a peptidase which preferentially cleaved certain methionyl peptides was localized in the fraction which precipitated between 50 and 75% saturation. A further purification of this enzyme was obtained by partially redissolving the precipitated protein from the preceding step in TM buffer which was 60% saturated with $(NH_4)_2SO_4$ (13). Under these conditions 80% of the enzyme remained insoluble. This precipitate was dissolved in TM buffer and thoroughly dialyzed against the same buffer. The resulting protein solution was used as the enzyme source for the experiments reported in this paper. At this stage of purification the peptidase was stable for two weeks when stored in TM buffer at 0-4°. More highly purified preparations lose activity rapidly unless stablized by Mn^{++}.

The rates of hydrolysis of the various peptides were monitored by

withdrawing aliquots of the reaction mixtures at appropriate times and assaying these for the appearance of free amino acids using the method of McDonald et al. (14). The trinitrobenzenesulfonic acid-copper reagent used in this assay reacts with free amino acids but not peptides, and consequently provides an accurate measurement of the initial rates of hydrolysis of peptides. The products of the enzymatic hydrolysis of the peptides were identified by comparison with standards after electrophoresis at pH 1.9 (3000 volts for 30 min.). The compounds separated by electrophoresis were located by reaction with ninhydrin reagent.

RESULTS

The initial rates of hydrolysis of the various dipeptides are shown in Table I. With the exceptions of N-formylmethionylalanine and glycyl-D-serine, all dipeptides tested were cleaved. However, the rates of hydrolysis of these compounds differed by as much as 100-fold. Methionylglycine, methionylalanine, methionylserine, and methionylmethionine were cleaved more rapidly than the other methionyl dipeptides or the dipeptides beginning with another amino acid. These observations indicate that this peptidase is selective for the hydrolysis of certain methionyl dipeptides. Furthermore, the failure to cleave glycyl-D-serine at a measurable rate suggests that the peptidase is specific for peptides composed of L-amino acids.

The action of this peptidase on tri-, tetra- and pentapeptides with N-terminal methionine is shown in Table II. The tripeptides were cleaved more slowly than the analogous dipeptides and larger peptides were not hydrolyzed at a measurable rate. Examination of the reaction products from the tripeptides by high voltage electrophoresis showed only undigested substrate and free amino acids. These results indicate that the rate limiting step in the hydrolysis of the tripeptides is the cleavage of the first peptide bond to generate a free amino acid and a dipeptide. The resulting dipeptides appear to be hydrolyzed rapidly enough to prevent measurable quantities of these compounds from accumulating. Essentially the same results as shown in

TABLE I

INITIAL RATES OF HYDROLYSIS OF DIPEPTIDES

Methionyl-X dipeptides	nmoles/min/mg	X-alanine dipeptides	nmoles/min/mg
Met-Gly	2900	Leu-Ala	650
Met-Ala	2800	Thr-Ala	580
Met-Ser	1700	Ala-Ala	500
Met-Met	1700	Ser-Ala	490
Met-Thr	780	Gly-Ala	480
Met-His	410		
Met-Tyr	400	Alanyl-X dipeptides	
Met-Glu	110	Ala-Met	740
Met-Val	70	Ala-Leu	220
Met-Leu	50	Ala-Gly	50
Met-Ile	45		
N-fMet-Ala	0	Glycyl-X dipeptides	
		Gly-Ala	430
		Gly-Ser	125
		Gly-Gly	25
		Gly-D-Ser	0

One µmole of substrate was added to TM buffer, pH 7.4, (total volume 0.9 ml) and this solution warmed to 37°. The reaction was started by addition of 0.1 ml of enzyme solution containing 60 µg of protein. Aliquots, 0.1 ml, were withdrawn 0, 2, 4, 6, 10, 20 and 30 minutes after addition of enzyme and placed into 0.4 ml of 0.1 N HCl to stop the reaction. The entire sample from each time period was then assayed for release of free amino acids as described under Materials and Methods. Duplicate samples were assayed for each time period.

Tables I and II were obtained when a more highly purified enzyme preparation was used.

TABLE II

INITIAL RATES OF HYDROLYSIS OF N-TERMINAL METHIONYL TRI, TETRA, AND PENTAPEPTIDES

Substrate	nmoles/min/mg
Met-Met-Ala	80
Met-Ala-Met	38
Met-Leu-Gly	32
Met-Ala-Ser	26
Met-Gly-Met	12
Met-Gly-Met-Met	0
Met-Ala-Ser-Asn-Phe	0

See Table I for assay conditions

The assay used in these determinations was not sensitive enough to allow estimation of the K_m value for methionylalanine. However, the initial rate of hydrolysis of this substrate was constant over a concentration range of 0.5 to 10 mM indicating that the K_m value for methionylalanine is less than 0.5 mM. In contrast the K_m value for methionylleucine was approximately 1 mM.

Unlike the ribosomal peptidase reported by Matheson and Dick (9) our enzyme appears to be a soluble protein. Under conditions of our assay, E. coli ribosomes did not catalyze the hydrolysis of methionylalanine nor did removal of ribosomes from crude homogenates reduce the level of peptidase activity in the fraction precipitating between 50 and 75% $(NH_4)_2SO_4$ saturation.

DISCUSSION

Information from studies of the N-terminal and second residues of E. coli proteins synthesized in vivo and in vitro allows certain prediction concerning the specificity of the enzyme involved in hydrolysis of N-terminal methionine

from newly initiated peptides (9,10,15). This peptidase should efficiently catalyze the hydrolysis of N-terminal methionine from peptides with the beginning sequences methionylalanine and methionylserine and should be less efficient in the cleavage of other N-terminal methionyl peptides. Since leucine and/or isoleucine and threonine are frequently found in the second position of native E. coli proteins (15), the proposed enzyme may be especially inefficient in catalyzing the hydrolysis of N-terminal methionine from peptides with the beginning sequences methionylleucine, methionylisoleucine, and methionylthreonine. Finally, since many native E. coli proteins contain either N-terminal alanine or serine, peptides beginning with these amino acids should be poor substrates for the enzyme as should peptides with N-terminal formylmethionine (7,9). Using a similar rationale,other investigators have examined E. coli preparations for peptidases which may be involved in the removal of N-terminal methionine from newly initiated proteins (9,10). A ribosomal peptidase reported by Matheson and Dick (9) appears to be favorably located for this function but is not highly specific for the hydrolysis of methionyl peptides. Furthermore, Vogt (10) has recently purified a soluble aminopeptidase (aminopeptidase I) from extracts of E. coli which has many properties in common with the ribosomal peptidase. The former enzyme is not thought to be involved in the cleavage of N-terminal methionine after initiation. Vogt (10) suggests that aminopeptidase I and the ribosomal peptidase are identical and that the binding of this peptidase to the ribosome is a result of the use of a low ionic strength buffer for ribosome isolation. At present the existance of a ribosomal peptidase which is specific for the hydrolysis of N-terminal methionine is uncertain; however, proteins which function in processes occurring on ribosomes are not necessarily permanently bound to the ribosomes. For instance, the E. coli deformylase (6), some of the protein factors involved in the initiation of protein synthesis (16), in peptide chain elongation (17), and in termination of protein synthesis (18), appear to be only transiently bound to ribosomes.

Based on the relative initial rates of hydrolysis of dipeptides, the peptidase examined in the present studies exhibits several of the properties predicted for the enzyme involved in the removal of N-terminal methionine after initiation of protein synthesis. This peptidase catalyzes the hydrolysis of methionylalanine and methionylserine more efficiently than that of methionylleucine, methionylisoleucine and methionylthreonine. It is relatively inefficient in the hydrolysis of dipeptides beginning with amino acids other than methionine and it does not promote the cleavage of N-formylmethionylalanine. Also, the K_m value for methionylalanine is less than 0.5 mM whereas this value for methionylleucine is 1 mM. These results are compatible with the predicted properties of the enzyme catalyzing the removal of N-terminal methionine. On the other hand, this enzyme was inefficient in the hydrolysis of methionyl tripeptides and inactive toward larger methionyl peptides. The latter observations may reflect the nature of the natural substrate for the enzyme involved in the removal of N-terminal methionine or may indicate that our enzyme is simply a peptidase which preferentially catalyzes the hydrolysis of certain methionyl dipeptides. If methionine is removed from bacterial proteins at an early stage of chain elongation as it appears to be from the newly initiated α-chains of rabbit hemoglobin (19) the proper substrate for the peptidase is a small peptidyl-tRNA bound to the ribosome surface. Whether our enzyme catalyzes the removal of N-terminal methionine from peptidyl-tRNAs is unknown, but this possibility is presently being investigated.

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