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THE THIOLESTERASE ACTIVITY OF LEUCINE AMINOPEPTIDASE

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SUMMARY

1. Leucine aminopeptidase (L-leucyl-peptide hydrolase, EC 3.4.1.1) has been shown to be capable of hydrolyzing leucine thiol ethyl ester at a rate 50 times greater than the rate for L-leucine-*p*-nitroanilide.

2. The pH optimum for the reaction is 8.6 and the K_m is $1.3 \cdot 10^{-3}$ M.

3. Both products of the reaction are capable of product inhibition. Alcohols also inhibit the thiolesterase activity of leucine aminopeptidase, but are less effective than mercaptans. $(NH_4)_2SO_4$ protects leucine aminopeptidase from inhibition by 2-mercaptoethylamine. This effect is not seen in combinations of ammonium sulfate and other mercaptans.

4. A new assay method has been developed which is useful for the measurement of thiolesterase activity of proteolytic enzymes. This method is based on the development of color by the reaction of liberated mercaptan with 5,5'-dithiobis-(2-nitrobenzoic acid).

5. These results extend the types of proteolytic enzymes which are capable of thiolesterase activity to include exopeptidases and metalloenzymes.

INTRODUCTION

It has been known for some time that some proteolytic enzymes are capable of hydrolyzing thiolesters derived from amino acids or peptides which are included in their specificities[1,2]. (R. B. Johnston, R. A. Ray and G. Luikhart, personal communication). However, none of the known thiolesterases are exopeptidases, nor do any of these enzymes contain metals at their active sites. Leucine aminopeptidase (L-leucyl-peptide hydrolase, EC 3.4.1.1) is both an exopeptidase and a metalloenzyme, and therefore appears to be an appropriate enzyme upon which studies could be performed to determine if the thiolesterase activity was a more general phenomenon of proteolytic enzymes than had been previously demonstrated.

While very few thiol ester derivatives of amino acids or peptides have been isolated from natural sources, this may be due to the lability of thiol ester bonds to both enzymatic and nonenzymatic hydrolysis in the cell. It is possible that amino

acid or peptide thiol esters may be of biological importance and, indeed, it has been demonstrated that the thiol ester grouping can function as an activating group for the synthesis of a peptide bond by papain[3]. This suggests the possibility of a role for amino acid thiol esters as intermediates in synthetic processes, *i.e.* transamidation, in the cell[4].

METHODS AND MATERIALS

Materials

Hog leucine amino peptidase was obtained from Worthington Biochemical Corp., the 5,5'-dithiobis-(2-nitrobenzoic acid) from Sigma Chemical Co., the 2-mercaptoethanol and 2-mercaptoethylamine from Swartz/Mann, the L-leucine-*p*-nitroanilide from Cyclo Chemical Co., and the mercaptoacetic acid (thiovanic acid) was from Evans Chemical Co. All other reagents were reagent grade.

Methods

The leucine thiol ethyl ester was prepared as described by Ray and Johnston[5], and the melting point agreed with the value reported.

The method employed for the hydrolysis of leucine thiol ethyl ester, except where otherwise specified was the following: 0.1 ml 0.1 M $MgCl_2$, 0.1 ml enzyme containing 1–10 μg protein, 3.0 ml 0.1 M Tris–HCl buffer (pH 8.6) and 0.1 ml 0.01 M 5,5'-dithiobis-(2-nitrobenzoic acid) were mixed in a cuvet of 1 cm light path. The reaction was initiated by the addition of 0.2 ml of freshly prepared 0.01 M leucine thiol ethyl ester, and the absorbance was monitored at 412 nm in a Gilford Model 2000 spectrophotometer which was controlled at 25 °C. Enzyme was omitted from the blank. The rate of reaction was determined from the slope of the recording obtained at 412 nm, assuming an extinction coefficient of 13 600 (ref. 6). Four rates can be obtained simultaneously using this spectrophotometer. The change in absorbance is directly proportional to the enzyme concentration over the range employed in these studies. The enzyme was prepared immediately before use by passing 50 μl (250 μg) through a 0.9 cm $\times$ 18 cm column of Sephadex G-25 in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.001 M $MgCl_2$.

A rapid and convenient semi-quantitative test for activity can be achieved by mixing one drop of each of the following reagents (0.1 M $MgCl_2$; 0.1 M Tris–HCl buffer (pH 8.6); 0.01 M 5,5'dithiobis-(2-nitrobenzoic acid); 0.01 M leucine thiol ethyl ester with one drop of the sample to be tested in a well of a spot plate. The development of a yellow color more intense than the blank is indicative of the presence of enzyme. Such tests have proven useful for locating activity in fractions from column chromatography and for preliminary, qualitative experimentation.

In those experiments which required a substrate other than leucine thiol ethyl ester, L-leucine-*p*-nitroanilide[7] was employed at a concentration of 1.6 mM in 0.033 M Tris–HCl buffer (pH 8.0) with 3.3 mM $MgCl_2$, at 37 °C. The reaction was followed at 405 nm. All inhibitor solutions were at pH 8.0.

RESULTS

The pH optimum for the hydrolysis of leucine thiol ethyl ester by leucine amino-

peptidase is 8.6 and is similar to that reported for the hydrolysis of leucyltyrosine[8] and leucinamide[9] by the same enzyme.

The K_m was found to be 1.3 mM graphically by plotting $1/v$ *vs* $1/[S]$. This value can be compared to a K_m of $5.7 \cdot 10^{-3}$ M reported for the hydrolysis of leucinamide at pH 8.4 and the value of $1.6 \cdot 10^{-3}$ M reported for L-leucine benzyl ester[8].

Ethanol and *n*-propanol, which are known inhibitors of leucine aminopeptidase[10], were tested for inhibition of the thiolesterase activity. The results are similar to those reported for the amidase activity of leucine aminopeptidase. The inhibition by high concentrations of alcohol is reversible by dilution with buffer.

The effect of products of the reaction on the thiolesterase activity is shown in Table I. The effect of mercaptans on the activity of the enzyme indicates that both

TABLE I

INHIBITION OF LEUCINE AMINOPEPTIDASE

The activity was measured by the hydrolysis of leucine-*p*-nitroanilide, and relative to the uninhibited sample set at 100%.

Inhibitor	*Concn. (mM)*	*Activity (%)*	*Activity (%)***		
			+ $(NH_4)_2SO_4$	*+ NH_4Cl*	*+ NaCl*
None	—	100	91	112	119
L-Leucine*	50	26	—	—	—
Ethanolamine	33	107	—	—	—
Mercaptoacetic acid	33	4	8	—	—
2-Mercaptoethanol	33	29	46	—	—
2-Mercaptoethylamine	33	0	105	114	4

* The inhibition by L-leucine was measured by means of the thiolesterase assay as described in methods and materials.

** The activities were measured in the presence of 0.2 M $(NH_4)_2SO_4$, NH_4Cl or NaCl (pH 8.0).

products of the reaction are inhibitors. It was not possible to test ethyl mercaptan directly for activity because it has very limited solubility in the aqueous systems employed in these tests. Several water-soluble, two-carbon mercaptans were therefore tested for inhibition. The use of these compounds precluded the use of the thiolesterase assay since they interfere with the accurate measurement of the liberated mercaptan. The inhibitory effect of mercaptans was therefore studied by measuring the hydrolysis of L-leucine-*p*-nitroanilide[7], the assumption being made that the mechanism of hydrolysis of the two substrates was essentially the same.

It appears that mercaptans are better inhibitors than the alcohols are, although it is necessary to realize that most of the tests of the inhibitory potency of the two classes of compounds were performed with different assay methods. A direct comparison can be made between ethanol amine and 2-mercaptoethylamine. Under the conditions of the test, the mercaptan completely inhibits the enzyme while the alcohol is without effect.

$(NH_4)_2SO_4$ has been reported to be an inhibitor of the hydrolysis of leucinamide by leucine aminopeptidase[10], but this compound was shown to have very little effect on the thiolesterase activity (Table I) under the conditions employed in these tests. It is of interest that 0.2 M $(NH_4)_2SO_4$ or NH_4Cl protects the enzyme from inhibition

by 2-mercaptoethylamine, while NaCl does not protect the enzyme. It, therefore, appears that NH_4^+ is responsible for the protective effect. A very weak protective effect may also be seen with 2-mercaptoethanol, while mercaptoacetic acid inhibits equally well in the presence or absence of ammonium salts.

The strong protective effect of ammonium salts against 2-mercaptoethylamine can be explained on the basis of a competition between NH_4^+ and the amino group of the mercaptan for a binding site, perhaps negatively charged, near the active site of the enzyme. If the more bulky mercaptan binds, it might block the entrance of the substrate into the active site. However, the small NH_4^+ may not have this effect.

Alternatively, it may be postulated that the negatively charge binding site may be near a sulfhydryl binding site. The presence of the positively charged NH_4^+ may drastically reduce the binding of the positively charged mercaptan while having relatively little effect on the neutral or acid mercaptans.

DISCUSSION

The results reported here demonstrate that leucine aminopeptidase is an efficient thiolesterase. It has long been known that this enzyme is capable of hydrolyzing the carbon–nitrogen bonds of amides[9] and Bryce and Rabin[8] have shown that the carbon–oxygen bonds of esters are also attacked by the enzyme. This report extends the known action of leucine aminopeptidase to include the carbon–sulfur bonds on thiol esters. Leucine aminopeptidase is the first exopeptidase and the first metal requiring proteolytic enzyme to be demonstrated to possess thiolesterase activity.

The method employed in this report is a new procedure for measuring the thiolesterase activity of proteolytic enzymes. With the proper substrate it should be applicable to other proteolytic enzymes which are capable of hydrolyzing thiol esters. The advantages of this method over the hydroxamate method[4] previously used are that it is a more convenient method and that it measures the formation of a product rather than the decrease in substrate. The thiol ester is a better substrate than L-leucine-*p*-nitroanilide, it is freely soluble in aqueous media and the resulting colored complex has a higher extinction coefficient than *p*-nitroaniline. These factors combine to make the thiolesterase assay 65 times a sensitive as the hydrolysis of L-leucine-*p*-nitroanilide.

Since the pH optimum, K_m and effect of alcohols and leucine on the rate of hydrolysis are similar to those previously reported for esters and amides, it appears that a similar mechanism of action is involved in the hydrolysis of these substrates.

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