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ENZYMIC CLEAVAGE OF THE BLOCKED AMINO TERMINAL RESIDUES OF PEPTIDES

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SUMMARY - The substrate specificity of an enzyme that removes some N-terminal blocked amino acids from blocked peptides has been further explored with several naturally-occurring peptides. Chloride ion is an effective modulator of enzyme activity. Although the relative efficiency of the enzyme varies considerably with different peptide substrates, in each case there was significant although less than quantitative release of the N-terminal blocked amino acid. The possible application of this enzyme to structural studies on polypeptides is evaluated.

In a previous communication (1) we described the preparation and some of the properties of an enzyme, acylpeptide hydrolase, from mature human red cells. This enzyme catalyzes the removal of an acyl amino acid residue from an acylated peptide substrate as described in the following equation:

$$\text{acyl} - \text{aa}_1 - \text{aa}_2 \ldots \text{aa}_n \longrightarrow \text{acyl aa}_1 + \text{aa}_2 \ldots \text{aa}_n$$

This enzyme preparation behaves as one band upon gel electrophoresis under non-denaturing conditions (1). Its substrate specificity is quite broad. Hence, acetylated N-terminal amino acid residues comprising -alanine, -glutamate, -serine, -phenylalanine, or -glycine are cleaved from acylated peptide substrates with varying efficiency. Formyl methionine and carbamyl glycine are also removed from N-terminal blocked peptides. Moreover, we find that the pH optima for some of these substrates differs. Thus, substrates with blocked acidic N-terminal residues have pH optima in the range of 6 but blocked amino acid residues with uncharged residues have pH optima in the range of 7-8. In addition, small (C_4-C_6) fatty acyl esters are also substrates for the enzyme.

The abbreviations used are: Ac, acetyl: f, formyl: MSH, melanocyte stimulating hormone: ACTH, adrenocorticotropic hormone.

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The presence of this enzyme was first reported by Narita and his colleagues (2) and subsequently by several other investigators (3-6). In the studies described below we demonstrate that on small peptide substrates the enzyme preparation is free of acylase, carboxypeptidase, or endopeptidase activities, and that on a polypeptide the amount of endopeptidase activity is marginal. Therefore, it seemed feasible to test some larger peptides as possible substrates for this enzyme and these results are reported herein.

METHODS AND MATERIALS

The preparation of acyl peptide hydrolase was described previously (1). For some experiments a further chromatographic step on QAE-cellulose (Bio-Rad, 0.9 x 10 cm) equilibrated in 25 mM potassium phosphate, pH. 6.5, was included. The enzyme was applied in the same buffer. After elution of the resin with 50 ml of buffer, the enzyme was eluted by addition of 0.2 M NaCl to the buffer. Protein concentration was determined by the Bio-Rad protein assay using BSA as a standard. For the peptides listed below, 4 μg of enzyme was used for deblocking at 37°C for the periods of time indicated.

The peptides α-MSH and ACTH fragment 34-39 as well as bovine serum albumin were purchased either from Sigma, K and K, or Bachem. Histone H-4 was a gift from Drs. V.G. Allfrey and R. Sterner. The detection of free amino groups after enzymic cleavage was determined by the Fluram assay (7). Acetylation of peptides with free N-terminal residues, i.e., ACTH fragment 34-39, was performed as described previously (1).

Enzyme Assays and Analysis of Product - Enzyme activity was assayed either against p-nitroanilide substrates or peptide substrates at approximately 4 mM concentration of each as described previously (1). Activity was linear with enzyme concentration, which varied depending upon the relative efficiency of the substrate. In studies on small peptides, the reaction was stopped by acidification with HCl to about pH 2. The solution was applied to a 0.6 x 5.0 cm column of Dowex-50X2, 200-400 mesh. The blocked amino acid corresponding to the N-terminus was eluted with 2 column volumes of water. After evaporation of this eluate in a Savant Concentrator, the residue was dissolved in 6 N HCl and heated at 110° for 16-20 hrs. After removal of HCl, the solution was adjusted to pH 2.2 and amino acid analysis was performed on a Dionex D-500 instrument.

For the estimation of the amount of endopeptidase on larger polypeptides, bovine serum albumin was treated with the enzyme for 20 hr at 25° and polyacrylamide gel electrophoresis was performed as described previously (8).

RESULTS AND DISCUSSION

Assay for Proteolytic Enzymes in the Preparation - In order to determine whether there were other proteolytic activities in the enzyme preparation, two small peptide substrates were treated extensively with the enzyme. There was no free phenylalanine released from the C-terminus of the acetylated ACTH fragment 34-39. This result excludes the presence of an activity similar to V8-protease in the preparation.

Table 1

ENZYMIC CLEAVAGE OF PEPTIDES

Substrate	N-Terminus	Incubation Time (Hrs)	% Deblocking
Ac-(Ala)$_3$	Ac-Ala	1	100
f-Met-Val	f-Met	40	50
α-MSH	Ac-Ser	20	65
Ac-ACTH(34-39)	Ac-Ala	4	85
Histone H-4[a]	Ac-Ser	20	69

The incubations were carried out at the following concentrations of peptides: Ac-(Ala)$_3$:4 mM, f-Met-Val:4 mM, α-MSH:0.24 mM, Ac-ACTH (34-39):3.4mM, and Histone H-4:0.16 mM at 37°C for the designated times. The extent of enzymic cleavage was determined as described in the text.

[a] Histone H-4 was digested twice with 1.5% trypsin for 48 hrs at 37°C prior to addition of the hydrolase.

Acetyl trialanine, one of the most efficient substrates for the hydrolase, was treated with the enzyme until cleavage was complete as judged by the response to Fluram (Table 1). Amino acid analysis of the reaction mixture indicated the presence of only dialanine (100% yield). There was no free alanine as judged by the amino acid analysis. This result excludes the presence of carboxypeptidase-like activity on the small substrate. In addition, there was no free trialanine. This finding indicates the absence of acylase-like activity. The fact that only dialanine was recovered is also indicative of the absence of endopeptidase activity in the enzyme preparation. This latter conclusion was tested directly by studies on a larger polypeptide, bovine serum albumin. After treatment of this protein with the hydrolase for 20 hrs at 25°C, analysis by polyacrylamide gel electrophoresis revealed the generation of a minor protein band corresponding to 2% of the undigested serum albumin. Thus, endopeptidase activity in the preparation is marginal.

Extended Cleavage of Small Peptide Substrates for the Hydrolase - As reported previously (1), acetyl serylleucine is only about 6% as active, on a relative basis, as the best substrate for the hydrolase, acetyl alanine p-nitroanilide. However, upon prolonged incubation with the enzyme (2 hrs) it

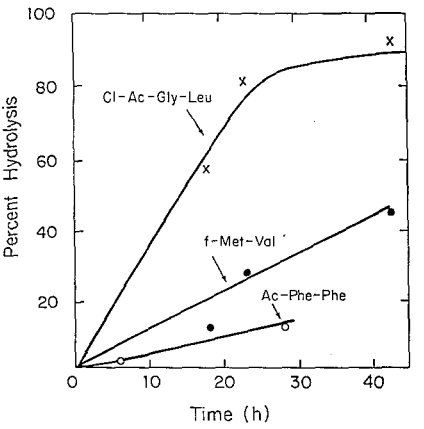

Fig. 1. Enzymic cleavage of blocked peptide substrates. Each substrate at a concentration of 4 mM was incubated with the hydrolase for the indicated periods in 0.05 M phosphate, p.H. 7.3. The extent of cleavage was determined by the Fluram assay as described in the text.

can be nearly completely cleaved to acetyl serine and leucine. This result suggested that the hydrolase might provide useful results with larger peptides even if they were relatively inefficient substrates. As shown in Fig. 1, Cl-Ac-Gly-Leu is nearly completely cleaved by the hydrolase after about 40 hrs of incubation with the enzyme. In addition, f-Met-Val is also extensively cleaved by the hydrolase, to the extent of about 50%, after the same time period of incubation. The relatively poor substrate Ac-Phe-Phe is cleaved to the extent of about 20%. Since the rates of cleavage for the latter two substrates are linear, perhaps either even longer incubation or treatment with larger amounts of enzyme would have led to a greater degree of cleavage. These findings, which demonstrate the stability of this enzyme under prolonged periods of incubation, are also consistent with our early reports on the activity of the hydrolase in the presence of trypsin or pronase (9).

Enzymic Cleavage of Other Blocked Peptides - To test the capability of the hydrolase in deblocking peptides of intermediate size, αMSH, a 13-residue peptide with acetyl serine at its NH_2-terminus, was chosen. Incubation of this blocked peptide with the hydrolase for 20 hrs led to the release of acetyl serine in 65% yield (Table 1). The acetyl serine was identified by amino acid analysis after Dowex-50 chromatography, acid hydrolysis, and amino acid analysis as described above.

A small fragment of ACTH, residues 34-39, was acetylated with acetic anhydride and the blocked peptide was subjected to the action of the hydrolase. As indicated in Table 1, acetyl alanine was released by the enzyme in 85% yield.

Native histone-H-4 is not a substrate for the hydrolase. However, after tryptic digestion, the hydrolase released acetyl serine in 69% yield from the NH_2-terminus of digested histone H-4. In these studies different concentrations of peptides were used. Due to lack of information on the relative efficiency of binding of these substrates to the enzyme, an absolute comparison of these peptides as substrates for the enzyme is not presently possible. Nevertheless, it is clear that each peptide is cleaved by the enzyme to the extent that information on the primary structure could be obtained.

The possible utility of this enzyme in structural studies on proteins is currently under study. In view of the problems encountered with peptides and proteins with blocked N-terminal residuess, perhaps this enzyme might have application in such cases. For peptides that are relatively inefficient substrates, it is possible that even a small degree of deblocking of the N-terminus would generate a fragment that could then be sequenced if the unblocked peptide did not interfere with the Edman degradation. If the parent peptide were small enough, the identity of the N-terminal amino acid could be inferred from knowledge of the amino acid composition of the peptide and the sequence remaining after enzymic deblocking. These possibilities are being evaluated on peptide fragments and on polypeptides.

Effect of Chloride on Enzyme Activity - Chloride is an activator of the hydrolase when an acetylated p-nitroanilide substrate such as acetyl alanine p-nitroanilide (AANA) is used as substrate (Fig. 2). This activation occurs up to a concentration of 0.1 M chloride and at higher concentration there is a slight decrease in activity. However, at such concentrations there is a marked inhibition of activity with the peptide substrates such as chloroacetyl-Gly-Leu (Fig. 2) or acetyl trialanine (data not shown). The

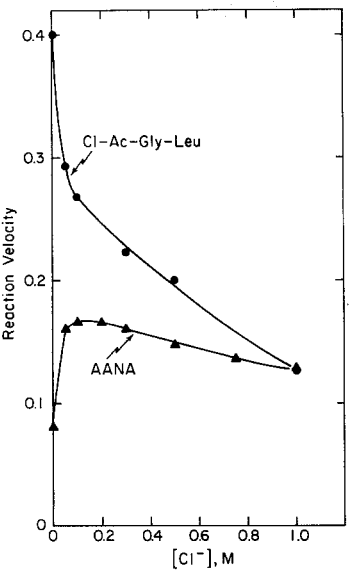

Fig. 2. The effect of chloride on enzyme activity. The indicated concentrations of chloride in 0.2 M potassium phosphate, pH. 7.3, were present during the assays. The substrate concentrations were 4 mM. AANA is acetyl alanine p-nitroanilide. The extent of hydrolysis was determined for each type of substrate as described previously (1).

effect of chloride has been demonstrated for a variety of peptidases including aminopeptidases (10), and for amylase (11). The role of chloride with metalloenzymes has been found to be due to the effect of anion on the metal ion and on the kinetic properties of the enzyme.

Possible Biological Role of the Enzyme - In addition to a possible application in structural studies on proteins a major question about this enzyme concerns its biological role. It has been postulated that the presence of an acetylated terminal residue on a protein marks that protein for degradation (12). Recently, it has been shown that the ubiquitin system, which is related to protein degradation, requires a free amino terminal residue (13).

It is conceivable that the blocking group is required for functional protein biosynthesis, i.e. to protect the free N-terminal amine during growth of the nascent chain. Thus, it is well established that in prokaryotic systems the initiating amino acid is a formyl methionine residue. In eukaryotic systems the picture is less clear. In some cases an unblocked methionine residue is present on an isolated protein and in other proteins the

acetylated terminus is retained. Indeed, Bradshaw and his colleagues have recently shown that if aldolase is isolated from the tissue in the presence of a protease inhibitor then the terminal residue is blocked. If the preparation of the enzyme is carried out in the absence of such an inhibitor, then the terminal blocking residue was removed (14). Perhaps the acyl peptide hydrolase that is described here is related to that process.

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REFERENCES

1. Jones, W.M., and Manning J.M. (1985) Biochem. Biophys. Res. Commun. 126, 933-940.
2. Narita, K., and Ishii, J. (1962) J. Biochem. 52, 367-373.
3. Gade, W., and Brown, J.L. (1978) J. Biol. Chem. 253, 5012-5018.
4. Schonberger, O.L., and Tschesche, H. (1981) Z. Physiol. Chem. 362, 865-873.
5. Witheiler, J., and Wilson, D.B. (1972) J. Biol. Chem. 247, 2217-2221.
6. Yoshida, A., and Lin, M. (1972) J. Biol. Chem. 247, 952-957.
7. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972) Science 178, 871-872.
8. Nigen, A.M., and Manning, J.M. (1978) J. Clin. Invest. 61, 11-19.
9. Jones, W.M., Manning, L.R., and Manning, J.M. (1986) Modern Methods of Protein Chemistry, in press, Plenum Press.
10. Kohm, K.-H. (1985) Arch. Biochem. Biophys. 239, 216-225.
11. Myrback, K. (1926) Z. Physiol. Chem. 159, 1.
12. Jornall, H. (1975) J. Theoret. Biol. 55, 1-12.
13. Hershko, A., Heller, H., Eytan, E., Kaklij, G., and Rose, I.A. (1984) Proc. Natl. Acad. Sci. 81, 7021-7025.
14. Lebherz, H.G., Bates, O.J., and Bradshaw, R.A. (1984) J. Biol. Chem. 259, 1132-1135.