219. *Acetaminoacylase and its Implication to Protein Biosynthesis*

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A working hypothesis that the N-terminal acetylated amino acid residue functions as the initiator of protein biosynthesis has been presented by Narita.¹⁾ We have studied ovalbumin synthesis using hen's oviduct system to verify above hypothesis and have reported evidence that acetylglycyl-tRNA served as the initiator of ovalbumin biosynthesis.^{2),3)} Another evidence to support our hypothesis has been presented by Liew, Haslett and Allfrey,⁴⁾ showing the participation of acetylseryl-tRNA in the initiation of histone synthesis in the regenerated rat liver. The majority of proteins, however, synthesized in cytoplasm of eukaryotes have free amino terminal residues. These facts can be reasonably explained by assuming the existence of an enzyme in cytoplasm which remove acetyl or acetaminoacyl group from the terminal acetylated polypeptides attached to ribosomes or from the detached acetylated proteins from ribosomes.

Thus we have attempted to search for such an enzyme from rat liver which is known to synthesize serum albumin ending acetyl-free N-terminal glutamic acid residue.⁵⁾ We used acetylmethionine and acetylmethionylthreonine (Ac-Met-Thr) as substrates to look for such an enzyme, since an acetamino acid deacetylase preferential for acetylmethionine and acetylthreonine was shown to be present in the supernatant fraction of rat liver homogenate.⁶⁾ The supernatant of the rat liver homogenate after centrifugation at 10,000×g for 20 min was found to contain an enzyme(s) to hydrolyze both the substrates. Therefore fractionation of the enzyme was carried out. The proteins possessing the enzyme activity were precipitated between 20 and 50% (NH₄)₂SO₄ saturation and the precipitates were dissolved in M/500 phosphate buffer of pH 7.2 containing 0.2 M NaCl. The supernatant after heating the solution at 60°C for 5 min was then chromatographed on a DEAE-cellulose column as shown in Fig. 1.

The enzyme specific for the acetamino acid (acetamino acid deacetylase) was clearly separated from the enzyme for the acetyldipeptide. The reaction mixture of Ac-Met-Thr with the latter enzyme contained acetylmethionine and threonine as the products. This

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Fig. 1. Elution of acetaminoacylase from a DEAE-cellulose column. Heat-treated supernatant of the rat liver homogenate containing 976 mg of protein (see the text) was placed on a 2.5×83 cm DEAE-cellulose column which had been equilibrated with 0.2 M NaCl-0.005 M sodium phosphate buffer, pH 7.2. After washing the column with a column volume of initial buffer, a linear gradient elution was carried out with 0.2 M-0.6 M NaCl in 0.005 M sodium phosphate buffer, pH 7.2. The column was finally washed with 2 M NaCl-0.005 M sodium phosphate buffer, pH 7.2. Fractions (about 15 ml) were assayed with Ac-Met-Thr (—) and acetylmethionine (----) as substrates. Protein (—) was determined by the absorbancy at 280 nm.

result suggested the possibility that the enzyme might be a carboxypeptidase. However, from the incubation mixture of acetylmethionyl-threonine methylester or acetylmethionylthreonine amide, acetylmethionine and the threonine ester or threonine amide were detected. Therefore the enzyme may not be a carboxypeptidase but an acetaminoacylase in nature. In order to confirm nature of the enzyme, effects of the enzyme on several synthetic acetylated peptides were examined. It is evident from Table I that the present enzyme is an acetaminoacylase which liberates acetyl amino acids from the N-acetyl-peptides. Table I further shows that the reaction rate is affected by the nature of the terminal acetyl amino acid; namely acetylmethionyl-peptides were the best substrates among examined. Formylmethionyl-threonine was also hydrolyzed by the enzyme in an appreciable rate.

Next, we have studied effects of the present acetaminoacylase on protein substrates. In this case the enzyme purified further by chromatography on a hydroxylapatite column followed by gel filtration on a Sephadex G-200 column, was used. As protein substrates, hen's ovalbumin having Ac-Gly-Ser-Gly-Ile-Ala- sequence at its N-

Table I. Relative Hydrolysis Rates of Acetylpeptides by the Enzyme

Enzyme activity was measured in a total volume of 0.5 ml buffered at pH 7.2 (0.05 M Na-phosphate) after incubation for 1 hour at 37.5°C. The relative values listed are based on the rate of hydrolysis of Ac-Met-Thr selected to be 100. Ac-Met-Thr was hydrolyzed at a rate of 0.72 μ mole/hour by this enzyme preparation

Substrate*	Relative hydrolysis rate	Main products
Ac-Met-Thr	100	Ac-Met and Thr
Ac-Met-Thr-OCH ₃	28.7	Ac-Met and Thr-OCH ₃
Ac-Met-Thr-NH ₂	65.0	Ac-Met and Thr-NH ₂
Formyl-Met-Thr	26.6	Formyl-Met and Thr
Ac-Gly-Ser	13.8	Ac-Gly and Ser
Ac-Leu-Ala-Gly	62.5	Ac-Leu and Ala-Gly
Ac-Phe-Gly-Phe	27.5	Ac-Phe and Gly-Phe
Ac-Ala-Leu	0	none
Ac-Gly-Asp-Val-Gly	0	none
Ac-Met	0	none
Ac-Gly	0	none

* Ac-Met-Thr-OCH₃ and Ac-Met-Thr-NH₂ represent respectively acetyl-methionylthreonine methylester and acetylmethionylthreonine amide.

terminus,⁷⁾ baker's yeast cytochrome c possessing Thr-Glu-Phe-Lys-sequence⁸⁾ and its acetylated derivative with ¹⁴C-acetic anhydride were used. When native ovalbumin was digested with the enzyme, small amounts of serine (0.027 mole/mole of ovalbumin), isoleucine (0.025 mole) and alanine (0.033 mole) appeared as the N-terminal groups by the dinitrophenylation method, suggesting that the terminal acetylglycine was liberated. DNP-Glycine seemed to be decomposed during acid hydrolysis. Almost identical results were obtained with denatured ovalbumin, suggesting that liberation of acetylglycine and several amino acids was not due to the protein conformation. These results indicate that the present acetaminoacylase preparation may contain an aminopeptidase as a contaminant, which affects on free N-terminal residue sequentially after the removal of the terminal acetylglycine. However, almost no threonine was released from the N-terminal position of baker's yeast cytochrome c even after denaturation. In contrast to the unmodified yeast cytochrome, radioactive acetylthreonine was liberated from the acetylated cytochrome, confirming that the enzyme is an acetaminoacylase in nature and that the contaminating aminopeptidase, if it were really present, is affected its action by neighbouring amino acid residues of the terminus. Another explanation on the above results is that the present enzyme possesses dual activities, acetaminoacylase and aminopeptidase, in a molecule. When homogeneous enzyme preparation is obtained, ambiguous properties of the enzyme described above will be clarified.

The present acetaminoacylase will act on the N-terminal acetylated nascent polypeptides attached to ribosomes during protein synthesis or the detached acetylated proteins from the ribosomes after the completion of the synthesis, if our assumption that protein biosynthesis in cytoplasm of eukaryotic organisms is initiated with acetylaminacyl-tRNA, was the case. Amino acids may be regenerated by the action of acetylaminic acid deacetylase from the released acylaminic acids and may be used again as materials for protein synthesis in cytoplasm. In the case of naturally occurring acetylated proteins at their termini, three possibilities can be considered. The one is that the cells synthesizing acetylated proteins do not contain such an acetaminoacylase.¹⁾ Cells to synthesize ovalbumin in hen's oviduct are probably this case, since the present rat liver enzyme hydrolyzed ovalbumin yielding acetylglycine in both native and denatured states. In fact, in the preliminary experiments, such enzyme activity in the 10,000×g supernatant of hen's oviduct homogenate was about only 1/7 of that in rat liver. The second is that the acetylated N-terminus is imbedded in the molecule even during the growing of the polypeptide chains on ribosomes, and thus the enzyme cannot be accessible to the acetylated terminus although the cells contain acetaminoacylase.²⁾ The third case is that the cells contain the enzyme and the acetylated terminus is exposed on the surface of the protein molecule but the completed molecule still possesses an acetyl at the terminus. Vertebrate heart muscle cytochromes c are probably this case, since the enzyme gave no influence on synthetic Ac-Gly-Asp-Val-Glu, as shown in Table I, which is the N-terminal peptide of cytochromes c from bovine, horse, etc., and since X-ray crystallographic analyses of horse heart and bonito cytochromes c demonstrated that the terminal peptide chains were exposed on the surface of their molecules.⁹⁾

Recent reports from several laboratories demonstrated that unsubstituted methionyl-tRNA_f participated in the initiation of polypeptide synthesis in cytoplasm from eukaryotic cells (reticulocytes, trout testis and wheat germ).¹⁰⁾⁻¹⁵⁾ These papers seem to indicate that the mechanism of protein chain initiation in cytoplasm may be different from specific cell to others.

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