STUDIES ON THE MECHANISM OF PROTEIN SYNTHESIS INCORPORATION OF ETHIONINE INTO α-AMYLASE OF *BACILLUS SUBTILIS*

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SUMMARY

1. α-Amylase containing ethionine was separated in crystalline form from the culture medium of the methionine-requiring mutant of *Bacillus subtilis*.

2. The sedimentation, viscosity and electrophoretical mobility of α-amylase containing ethionine were compared with those of normal enzyme. The molecular weight of α-amylase was estimated as 55,000.

3. The peptides containing methionine and ethionine were separated by column chromatography from a tryptic hydrolysate of α-amylase containing ethionine, and the amino acid composition of these peptides was estimated.

4. It was concluded that ethionine can be incorporated into the normal peptide-bond sequence of α-amylase, and that α-amylase containing ethionine has the same physico-chemical properties and enzyme activity as those of normal protein.

INTRODUCTION

Evidence for incorporation of certain amino acid analogues, such as *p*-fluorophenylalanine[1,2], thienylamine[2], ethionine[3], azatryptophan[4,5] and selenomethionine[6], into cellular protein has been presented. It has also been observed that synthesis of some enzymes and cell growth are inhibited by incorporation of such amino acid analogues into cells[2,5].

The following plausible mechanism can be proposed to explain these results. For example, amino acid analogue may become incorporated into the normal peptide-bond sequence of normal protein in exchange for normal amino acid. If the amino acid moiety replaced is essential for activity, the protein may not possess the same biological activity as that of normal protein. Or possibly, amino acid analogue may only participate in the synthesis of an "abnormal" protein, and synthesis of an "abnormal" protein or the "abnormal" protein itself may inhibit normal protein synthesis. Alternatively, it is also possible that these two mechanisms are co-operating in living organisms.

In order to approach the mechanism of protein synthesis, it will be important to distinguish these possibilities. For this purpose, the protein containing amino

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acid analogue will have to be isolated in a pure form, and it will be necessary to study its amino acid pattern, biochemical activity and physico-chemical properties.

In a previous paper[7], using the *Bacillus subtilis*–α-amylase system, it has been shown that some parts of methionine in the α-amylase molecule can be replaced by ethionine, and α-amylase containing ethionine has the same enzyme activity as that of normal enzyme. In the present work, the peptides containing methionine and ethionine were separated by column chromatographic technique from a tryptic hydrolysate of α-amylase containing ethionine, and the amino acid composition of these peptides was determined. It was concluded that ethionine can be incorporated into the normal peptide-bond sequence of α-amylase molecule by uniformly exchanging with methionine during the process of protein synthesis. Some physico-chemical properties of α-amylase containing ethionine were compared with those of normal enzyme.

MATERIALS AND METHODS

Bacterials strains used

Bacillus subtilis strain K and the methionine-requiring mutant (strain K-Met) were used in this experiment. Strain K-Met was obtained from the wild type parent by M. KOHIYAMA using the penicillin-screening method at the Institute of Applied Microbiology of the University of Tokyo.

Materials used

Soluble starch purchased from the Wako Pure Chemical Industries (Japan), Trypsin (2 × Cryst. 50 % $MgSO_4$) purchased from the Nutritional Biochemical Corporation and Dowex-50-X2 purchased from the Dow Chemical Company were used in this work. Whatman No. 1 filter paper was used for paper chromatography and paper electrophoresis.

Culture method

The bacteria were grown on the surface of the synthetic medium containing 7 % soluble starch, 0.9 % sodium citrate, 1.3 % $(NH_4)_2HPO_4$, 0.1 % KCl, 0.05 % $MgSO \cdot 7H_2O$ at pH 7.0 and 37°. For growth of the methionine-requiring mutant, the basal medium was supplemented with methionine or methionine and ethionine.

Isolation and crystallization of α-amylase

When the α-amylase activity of the culture medium reached a maximum, the cells were filtered off through cloth. The method employed for crystallization of the enzyme from the filtrate is similar to HAGIHARA's method[8]. The procedures are shown in Scheme 1. About 50–100 mg of the lyophilized enzyme powder could be obtained from 1 l of the culture medium after recrystallization three times.

Normal α-amylase was prepared from the basal culture medium using strain K or from the culture medium containing 20 mg/l of L-methionine using strain K-Met. α-Amylase containing ethionine was prepared from the culture medium containing 5 mg/l of L-methionine and 20 mg/l of DL-ethionine using strain K-Met. ^{35}S-labelled α-amylase was prepared from the culture medium containing 1.5 mC/l of ^{35}S using strain K.

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SCHEME I

CRYSTALLIZATION OF α-AMYLASE

Filtered culture medium
| add 0.5 *M* $CH_3COO\frac{1}{2}Ca$ (200 ml/l)
| pH 6.5 with 2 *N* NaOH
| filter with Hyflosupercel (2 g/l) using a Büchner funnel
Filtrate
| add $(NH_4)_2SO_4$ (420 g/l of filtrate)
| stir at room temperature for 1 h
| filter with Hyflosupercel (2 g/l) using a Büchner funnel
Filter cake
| suspend in 0.01 *M* $CH_3COO\frac{1}{2}Ca$ (20 ml/l of culture medium)
| pH 9.0 with *N* NH_4OH
| extract at room temperature
| repeat twice
Extract
| dialyse against 0.005 *M* $CH_3COO\frac{1}{2}Ca$
| 2–3 days at 0°
Dialysate
| add acetone under stirring at 0° (final 65 % acetone, v/v)
| centrifuge at — 5° to 0°
Precipitate I
| suspend in a small volume of 0.01 *M* $CH_3COO\frac{1}{2}Ca$
| leave for 1 night at 0°
| add acetone (40 % v/v)
| centrifuge at — 5° to 0°
Precipitate II
| wash with 30 % acetone containing 0.005 *M* $CH_3COO\frac{1}{2}Ca$
| centrifuge at — 5° to 0°
| repeat 3 times
Precipitate III
| dissolve in a small volume of water containing
| $Ca(OH)_2$, pH 10
| centrifuge at 0°
Supernatant
| pH 6.5 with 0.5 *M* CH_3COOH
| leave for 1 night at 0°
| centrifuge at — 5° to 0°
Crystalline α-amylase

For recrystallization, crystalline α-amylase was dissolved in a small volume of water and treated as Precipitate III.

Assay of α-amylase activity

The enzyme activity was measured in the reaction mixture containing 1.0 % soluble starch, 0.05 *M* NaCl, 0.01 *M* calcium acetate, 0.1 *M* acetate buffer (pH 5.6) and enzyme at 40°. Amylase activity was represented as Wohlgemuth's unit ($D_{40°}^{30'}$)[9].

Measurements of sedimentation and viscosity

Ultracentrifugation experiments were carried out in a Spinco Model-E centrifuge at a speed of 59,780 rev./min and at room temperature. The concentration of the protein was 0.7 %, and the solvent employed was acetate buffer at pH 6.0 and 0.2 ionic strength.

Viscosity, at a number of different concentrations ranging from 0.3 % to 0.7 % of the protein, was measured in the same solvent using the Ostwald viscosimeter at 20°.

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Paper electrophoresis

Paper electrophoresis was carried out in the solvent of 0.1 *M* tris buffer at pH 8.2 and 8.8 under constant current (0.62 mA/cm) at room temperature for 20 h.

Hydrolysis of denatured α-amylase with trypsin

α-Amylase was denatured by incubation at 20° for 20 h at a concentration of 1 % in 0.1 *N* HCl. The rate of the tryptic hydrolysis of denatured α-amylase was determined at 25°. The substrate was presented at a concentration of 0.5 % in 0.1 *M* phosphate buffer at pH 7.8, and the concentration of trypsin (50 % $MgSO_4$) was 0.005 %.

Separation of peptides by column chromatography

The separation of the peptides was effected on 150 × 0.9 cm column of Dowex-50-X2, operated in the Na form, by procedures identical to those described by HIRS, MOORE AND STEIN[10]. The mixing chamber had a volume of 720 ml.

Quantitative estimation of amino acid

The peptides were hydrolysed completely in 6 *N* HCl at 110° for 24–28 h. The amino acid composition was determined by the method described by LEVY[11]. The ratio between methionine content and ethionine content in α-amylase molecule and cellular protein, was estimated by the same procedure.

Measurements of radioactivity

Fractionated samples were evaporated under infrared lamp and assayed, using a gas-flow counter (Nuclear Measurement Model PC-3A).

RESULTS

Effect of ethionine on bacterial growth and α-amylase production

Strain K in the basal medium and the methionine-requiring mutant (strain K-Met) in the basal medium supplemented with 20 mg/l of L-methionine, reached the stationary phase after an incubation of about 3 days. After a further incubation of 1 or 2 days, the α-amylase activity of the culture medium reached a maximum (4,000–6,000 $D_{40°}^{30'}$/ml). Growth of the both strains and the production of α-amylase exposed to 20 mg/l of D,L-ethionine were not inhibited. Strain K-Met was able to grow in the basal medium supplemented with 5 mg/l of L-methionine and 20 mg/l of D,L-ethionine but at a slower rate, an incubation of about 5 days being necessary to reach the stationary phase, and the α-amylase activity of the culture medium reached the same activity, *viz.* 4,000–6,000 $D_{40°}^{30'}$/ml. After growth in this medium, a back-mutation of strain K-Met into the wild-type parent strain K, did not take place. Strain K-Met could not grow in the basal medium supplemented with only ethionine.

α-Amylase prepared from the culture medium containing methionine and ethionine, contains ethionine as its molecular constituent.

Physico-chemical properties of α-amylase containing ethionine

Schlieren patterns of α-amylase containing ethionine and those of normal

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α-amylase show single, symmetrical sedimentation boundaries. This fact indicates a high degree of homogeneity in the both cases. The sedimentation constant ($S_{20,w}$) of α-amylase containing ethionine estimated from the experimental results is 4.25 *S*, and that of the normal enzyme is 4.22 *S*. These two values reconcile well within experimental error.

Intrinsic viscosity of the both α-amylases also reconcile well, *viz.* $[\eta] = 5.4 \pm 0.1$ ml/g.

From the sedimentation constant and the intrinsic viscosity ($S_{20,w} = 4.25$) and $[\eta] = 5.4$), together with an assumed value for partial specific volume 0.745 ml/g, and $\Phi' \cdot P^{-1} = 2.6 \cdot 10^6$, the molecular weight of α-amylase may be calculated from the MANDELKERN–FLORY equation[12]: a value of 55,000 was obtained. The molecular weight of α-amylase of another strain of *Bacillus subtilis* was estimated as about 45,000 from the unimolecular layer method[13].

Patterns of paper electrophoresis at pH 8.2 and 8.8, indicate the same electrophoretical mobilities of the both proteins as well as their homogeneity.

Amino acid pattern of α-amylase containing ethionine

The course of the tryptic hydrolysis of acid-denatured α-amylase at pH 7.8 and 25° is indicated by the curve shown in Fig. 1. It will be seen that the reaction proceeds almost to completion within a period of 5 h, and that, as measured by the increase of ninhydrin-reactive groups, α-amylase molecule is split into about 50 peptides. This result reconciles well with the fact that α-amylase has about 50 moles of lysine and arginine per mole[14]. Native α-amylase was stable towards tryptic digestion.

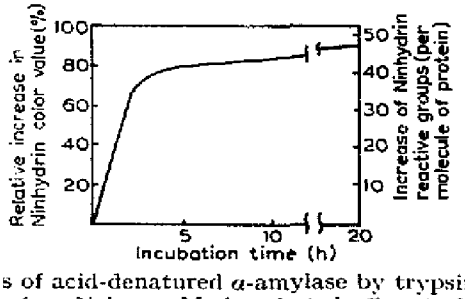

Fig. 1. The rate of hydrolysis of acid-denatured α-amylase by trypsin at 25°. The substrate was presented at a concentration of 0.5 % in 0.1 *M* phosphate buffer at pH 7.8, and the concentration of trypsin (50 % $MgSO_4$) was 0.005 %.

In view of the result shown in Fig. 1, a hydrolysate obtained after exposing acid-denatured α-amylase containing ethionine to the action of trypsin for 20 h was chromatographed on a 150 × 0.9 cm column of Dowex-50-X2. The effluent curve is shown in Fig. 2. Of these peaks, those marked I, II and III contained methionine and ethionine. From the fact that the corresponding peaks obtained from a hydrolysate of ^{35}S-labelled α-amylase by the same procedure showed no radioactivity, it was assumed that no other peaks contained sulphur-containing amino acid.

From the sulphur content of α-amylase (0.017 ± 0.002 mg/mg protein N) and its molecular weight, together with the fact that α-amylase has neither cysteine nor cystine[7,14], it is shown that one mole of α-amylase contains 4 moles of methionine or methionine and ethionine. So it might be expected that four peptides containing methionine and ethionine would be separated. But the fourth peptide could not be

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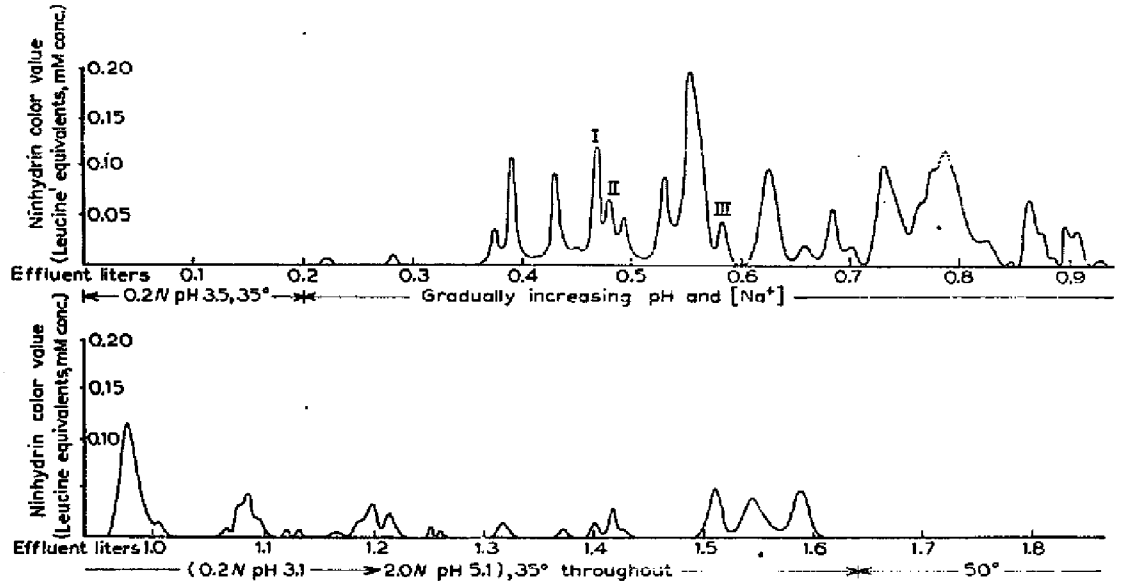

Fig. 2. The peptides in a 20-h tryptic hydrolysate of acid-denatured α-amylase containing ethionine. Chromatography of a hydrolysate from 40 mg of protein was carried out on a 130 × 0.9 cm column of Dowex-50-X2. The effluent was collected in 2-ml fractions. Aliquots (0.5 ml) were removed for analysis by the ninhydrin method.

TABLE I

AMINO ACID COMPOSITION OF PEPTIDES CONTAINING METHIONINE AND ETHIONINE

Amino acid	*Peak* I		*Peak* II		*Peak* III	
Methionine	0.64	1.00	0.61	1.00	0.60	1.00
Ethionine	0.36		0.39		0.40	
Aspartic acid	5.0_6		3.0_5		$11._6$	
Glutamic acid	2.0_6		2.8_0			
Glycine	1.8_8		2.0_5		3.2_5	
Alanine	4.2_0		1.8_5		1.0_2	
Valine	1.0_9		2.1_6		4.2_8	
Leucine and isoleucine	1.9_0		4.1_8		1.8_0	
Serine	1.9_5		0.0		0.9_6	
Threonine	0.9_7		1.1_8		1.8_0	
Proline	0.9_4		0.9_0		1.7_0	
Lysine	1.1_4		0.0		0.0	
C-terminal	Lysine		Arginine		Arginine	
N-terminal	*		*		Valine	
Yield of peptide	80%		50%		40%	

The composition of the peptides containing methionine and ethionine is expressed in terms of the molar ratios of the constituent amino acids, taking the amount of methionine and ethionine as 1.00.

* Not determined.

eluted out by the procedure, probably owing to the fact that the peptide contains aromatic amino acid residues as its molecular constituent.

The quantitative amino acid analysis summarized in Table I, indicated that the peaks marked I, II and III have the same molar ratio of methionine to ethionine (methionine : ethionine = 0.62 : 0.38). The ratio of methionine content to ethionine content of the whole α-amylase molecule containing ethionine is 0.61 : 0.39. So the ratio in the fourth peptide, which could not be determined directly, must be the same as that of others.

The results described above indicate that all four methionines have an equal chance of being replaced by ethionine in the α-amylase molecule, thus excluding the possibility that one or two particular methionine moieties will be preferentially replaced.

The ratio of methionine to ethionine in cellular protein is 0.59 : 0.41.

DISCUSSION

No work has yet been reported on the mechanism of incorporation of amino acid analogues into protein, and it has not yet been decided whether or not the protein containing amino acid analogues has biological activity.

In the present work, it has been established that ethionine can be incorporated into normal peptide-bond sequence of α-amylase by uniformly exchanging with methionine during the process of protein synthesis. Thus the process of protein synthesis permits ethionine to replace methionine.

Concerning the physico-chemical properties, no difference could be detected between the sedimentation velocity, viscosity and electrophoretical mobility of α-amylase containing ethionine and those of normal enzyme. As was shown in a previous paper[7], the enzyme activity of α-amylase containing ethionine is same as that of normal enzyme (80,000–90,000 $D^{30'}_{40^\circ}$/mg protein N in the both cases). So in the case of α-amylase, ethionine is sufficiently similar to methionine to have the same physico-chemical properties and biological activity. But ethionine can not be used as a substitute for methionine in the growth of the methionine-requiring mutant.

It has been reported that ethionine has an inhibitory effect on the formation of ribonucleic acid at higher concentrations[15]. In the case of *Bacillus subtilis*, ethionine had no effect on cell growth and the formation of α-amylase.

This report presents the first evidence for incorporation of an amino acid analogue into a normal peptide-bond sequence of normal protein. There is no reason, however, to postulate that all amino acid analogues are incorporated into protein by the same mechanism, or that protein containing other kinds of amino acid analogues has the same biological activity as that of normal protein. Further investigations must be carried out, using other types of amino acid analogues and other types of protein.

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CONTRIBUTION OF HYDROURACIL AND ITS DERIVATIVES TO PYRIMIDINE BIOSYNTHESIS

II. MECHANISM STUDIES

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SUMMARY

In an attempt to elucidate the mechanism of RNA formation from CβA and HU the relative incorporation of a number of derivatives was determined. From the data it is clear that the ribotides of CβA and HU are much superior to the parent compounds and to orotate or CO_2 as precursors of RNA. The ribosides of CβA and HU were incorporated somewhat better than the parent compounds.

To clarify the intermediate steps in the sequence, the appearance of [^{14}C]CβA and [^{14}C]HU into their ribotides was demonstrated. A reaction involving DPN and HURP to give URP was demonstrated and attempts to purify the system were made. Apparently, TPN and/or the flavins are not involved.

Evidence for the cyclization of CARP to HURP was obtained with the suggestion that a more exergonic condition than simple reversal of hydrolysis is required.

Abbreviations used in this paper are as follows: HU, 4,5-dihydrouracil; CβA, carbamoyl-β-alanine; C-Asp, carbamoyl aspartate; URP, uridylic acid; HURP, 4,5-dihydrouridylic acid; CAR, carbamoyl-β-alanine riboside, CARP, carbamoyl-β-alanine ribotide; HUR, 4,5-dihydrouridine; RNA, ribonucleic acid; DPN, TPN, di or triphosphopyridine nucleotide; DPNH, TPNH, reduced di or triphosphopyridine nucleotide.

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