INCORPORATION OF METHIONINE BY A SOLUBLE ENZYME SYSTEM FROM ESCHERICHIA COLI

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Summary

A soluble fraction from Escherichia coli B was found to incorporate methionine into 95°C CCl_3COOH-insoluble fraction. The incorporation required methionyl-tRNA synthetase, methionine tRNA, ATP, Mg^{2+} and bovine milk casein. The casein could be replaced by arginylated bovine serum albumin and arginylated bovine α-lactalbumin. A mixture of 19 amino acids other than methionine and GTP had no effect on the incorporation. KCl was rather inhibitory. Puromycin, RNase A and trypsin inhibited the incorporation, while DNase I did not. The soluble fraction also incorporated the methionyl moiety of methionyl-tRNA. This incorporation was not affected by the addition of free methionine.

Introduction

Since the first description of the incorporation of arginine into 95°C CCl_3COOH-insoluble fraction by a soluble system from rat liver (1), the occurrence of similar soluble systems in various eukaryotes has been reported (2-8), and attributed to the presence of a soluble enzyme, arginyl-tRNA:protein arginyl transferase (4). A similar incorporation of leucine and phenylalanine by soluble fractions from prokaryotes (9-11) was also attributed to the participation of another soluble enzyme of the similar type, leucyl-, phenylalanyl-tRNA:protein leucyl, phenylalanyl transferase (12). The arginyl transferase catalyzed the transfer of the arginyl moiety of arginyl-tRNA to the amino terminals of proteins having either aspartic or glutamic acid residues at the terminals (4, 13, 14). Similarly, the leucyl, phenylalanyl transferase catalyzed the transfer of the leucyl or phenylalanyl moiety from tRNA to the amino terminals of proteins having arginine or lysine residues at the terminals (11, 15).

The arginyl transferase has been found only in eukaryotes and the leucyl, phenylalanyl transferase only in prokaryotes. These two transferases are the only aminoacyl-tRNA:protein aminoacyl transferases thus far identified. Recently, we observed the incorporation of methionine into 95°C CCl_3COOH-insoluble fraction by the 105,000 x g supernate from Escherichia coli B. Some characteristics of this incorporation are described below.

Materials and Methods

L-[1-^{14}C]Methionine (60 Ci/mol), L-[methyl-^{14}C]methionine (56 Ci/mol), L-[U-^{14}C]leucine (342 Ci/mol) and L-[U-^{14}C]phenylalanine (522 Ci/mol) were purchased from The Radiochemical Center, England. Bovine casein (Purified) was purchased from Sanko Junyaku Co. Bovine α-casein, bovine serum albumin (Fraction V) and DNase I were from Sigma Chemical Co. A mixture of various tRNA's from Escherichia coli B was obtained from General Biochemicals. Escherichia coli B, harvested at the mid-log phase, was donated by Ajinomoto Co. and non-sterilized bovine skim milk by Morinaga Milk Co.

The casein and α-casein were further purified by the butyl alcohol method of Morton (16) before use . After purification both caseins could be dissolved up to 30 mg/ml in 50 mM Tris-HCl, pH 8.5. α-Lactalbumin was purified from skim milk by the successive application of the methods of Aschaffenburg (17) and Richter et al. (18). Arginylation of the α-amino groups at the amino terminals of the albumin and α-lactalbumin was performed according to Soffer using [^{12}C]arginine (14). One mol of the albumin and α-lactalbumin received 0.55 and 0.66 mol of the arginine, respectively, as estimated from separate runs done at the same time using [^{14}C]arginine. The casein, α-casein, albumin, α-lactalbumin, arginylated albumin and arginylated α-lactalbumin were extensively dialyzed against a large excess of 50 mM Tris-HCl, pH 8.5, before used.

Methionyl-tRNA synthetase was partially purified from Escherichia coli B according to Littauer (19). The specific activity of the synthetase was 6.2 nmol/min/mg. A mixture of aminoacyl-tRNA synthetases including the methionyl-, leucyl- and phenylalanyl-tRNA synthetases was prepared from Escherichia coli B by the method of Zubay (20). The specific activities of methionyl-, leucyl- and phenylalanyl-tRNA synthetases were 2.5, 1.2 and 2.6 nmol/min/mg, respectively. The methionyl-tRNA synthetase and the mixture of aminoacyl-tRNA synthetases were practically free of the activity for the incorporation of the radioactivity of methionine described below. Methionine tRNA was partially purified from commercial baker's yeast by the method of Holley et al. (21). [^{14}C]Methionyl-tRNA was prepared by the method of Littauer (19) using the L-[1-^{14}C]methionine, methionyl-tRNA synthetase and methionine tRNA. One mg of the tRNA was charged with 0.73 nmol of the methionine.

All operations for preparation of the supernates from Escherichia coli B were performed at 4°C. The wet cells (5 g) were mixed with 10 g of Al_2O_3, homogenized with mortar and pestle and suspended for 30 min in 28 ml of 20 mM Tris-HCl, pH 7.8, containing 5 mM magnesium acetate and 5 mM 2-mercaptoethanol. The suspension was first centrifuged at 20,000 x g for 20 min and then the little turbid supernate (20,000 x g supernate) was centrifuged at 105,000 x g for 2 h. The upper half portion of the clear supernate was taken as 105,000 x g supernate I. Similarly, the upper half portion of the clear supernate obtained by another centrifugation

of the supernate I under the same conditions was taken as 105,000 x g supernate II. All the supernates were dialyzed overnight against 2 l of 10 mM Tris-HCl, pH 7.8, containing 5 mM 2-mercaptoethanol before used.

The standard incubation mixture for the incorporation of the radioactivity of methionine into 95°C CCl_3COOH-insoluble fraction contained 20 μmol of Tris-HCl, pH 9.0, 5 μmol 2-mercaptoethanol, 4 nmol L-[1-^{14}C] methionine, 500 μg casein, 60 μg methionyl-tRNA synthetase, 600 μg methionine tRNA, 300 nmol ATP, 1 μmol magnesium chloride and 20 μl of the dialyzed supernate II in a total volume of 100 μl. The mixture was incubated for 40 min at 37°C and the incorporated radioactivity was determined on a 50 μl aliquot by the procedure of Mans and Novelli (22) unless otherwise indicated. Protein was assayed by the method of Lowry *et al.* (23).

Results

All of the supernates from Escherichia coli B examined here incorporated the radioactivity of the [^{14}C]methionine, leucine and phenylalanine into 95°C CCl_3COOH-insoluble fraction as shown in Table I. The 105,000 x g supernate I and II used here were the upper half portions of the clear supernates from the 105,000 x g centrifugations and only these portions were taken in order to avoid ribosomal contamination even when nothing was sedimented during centrifugation. The incorporation of the radio-

Table I

Incorporation of radioactivity of [^{14}C]methionine, leucine and phenylalanine by fractions from Escherichia coli B

	Activity pmol/min/ml supernate		
	As methionine	Leucine	Phenylalanine
20,000 x g Supernate	84.5 (6.82)	501 (40.4)	187 (15.1)
105,000 x g Supernate I	62.1 (6.64)	361 (38.6)	152 (16.3)
105,000 x g Supernate II	47.2 (6.85)	297 (43.1)	128 (18.5)

The standard procedure described in the Materials and Methods was used for the assay of the incorporation of the radioactivity from methionine except that 20 μl of the supernate II was replaced with 10 μl of each dialyzed supernate. For the assay of the incorporation of leucine or phenylalanine, the [^{14}C]methionine, methionyl-tRNA synthetase and methionine tRNA were further replaced with 4 nmol of L-[U-^{14}C]leucine (diluted to 86 Ci/mol with [^{12}C]leucine) or L-[U-^{14}C]phenylalanine (diluted to 250 Ci/mol with [^{12}C] phenylalanine), 36 μg of the mixture of aminoacyl-tRNA synthetases and 600 μg of the mixture of tRNA's, respectively. The values in the parentheses indicate the specific activities expressed in pmol/min/mg.

activity of methionine decreased with the three successive centrifugations similarly to those of leucine and phenylalanine, possibly catalyzed by the soluble enzyme, leucyl, phenylalanyl transferase, while the specific activity remained constant through the 105,000 x g centrifugations. These results exclude the contamination with ribosomal particulates.

Requirements for the incorporation of methionine by the supernate II and effects of some additions on the incorporation were shown in Table II.

Table II

Requirements for the incorporation of methionine

	Relative activity %
Complete I	100
- 105,000 x g supernate II	5
+ the supernate II exposed to 90°C for 1 min, 20 µl	5
- L-[1-^{14}C]methionine	0
+ L-[methyl-^{14}C]methionine, 4 nmol	92
- casein	3
- 2-mercaptoethanol	87
- methionyl-tRNA synthetase	69
- methionine tRNA	10
- ATP	0
- Mg^{2+}	1
- above four components	0
- L-[1-^{14}C]methionine + [^{14}C]methionyl-tRNA, 600 µg	47
+ L-[^{12}C]methionine, 4 nmol	47
+ 19 amino acids other than methionine, 4 nmol each	100
+ GTP, 60 nmol	96
+ KCl, 9 µmol	54
+ puromycin, 22 µg	25
+ DNase I, 2 µg	96
+ RNase A, 2 µg	5
+ trypsin, 4 µg	1
Complete II	96
Complete III	85

The compositions of the incubation mixtures for complete I and II were the same as that of the standard incubation mixture. The incubation mixture for complete III contained 36 µg of the mixture of aminoacyl-tRNA synthetases and 600 µg of the mixture of tRNA's in place of the methionyl-tRNA synthetase and methionine tRNA, respectively. The mixture for complete II was further incubated with 10 µg of RNase A for 40 min at 37°C after the standard incubation for 40 min. Other manipulations for the assay were performed as described in Materials and Methods.

Exposure of the supernate II to 90°C for 1 min resulted in an almost complete loss of the activity. Practically the same level of incorporation was observed even when L-[methyl-^{14}C]methionine was used instead of the [1-^{14}C] methionine. This indicates that the incorporated moiety is methionine rather than a portion of the methionine molecule. The addition of casein was essential to this incorporation. The incorporation was slightly dependent on the presence of 2-mercaptoethanol, if at all.

The incorporation of methionine was dependent on the presence of methionyl-tRNA synthetase, methionine tRNA, ATP and Mg^{2+} which are required for the methionyl-tRNA synthesizing system (19). In the absence of the four components, no incorporation proceeded but incorporation was substantially restored by the addition of [^{14}C]methionyl-tRNA in place of the [^{14}C] methionine. The restored incorporation was not affected by the addition of [^{12}C]methionine. These results indicate that the incorporation of

Table III

Effects of proteins on the incorporation of methionine and leucine

	Activity pmol/min/ml supernate	
	Methionine	Leucine
Complete	0.0	2.53
+ casein, 300 μg	42.4	270
+ α-casein, 300 μg	48.6	244
+ albumin, 300 μg	3.5	23.8
+ α-lactalbumin, 300 μg	2.4	10.4
+ arginylated albumin, 300 μg	24.9	352
+ arginylated α-lactalbumin, 300 μg	60.8	227

The standard incubation mixture lacking the casein was used as the complete mixture for the incorporation of methionine except that the methionyl-tRNA synthetase and methionine tRNA were replaced with 36 μg of the mixture of aminoacyl-tRNA synthetases and 600 μg of the mixture of tRNA's, respectively. In the complete mixture for the incorporation of leucine, the [^{14}C]methionine was further replaced with 4 nmol of L-[U-^{14}C]leucine (diluted to 86 Ci/mol with [^{12}C]leucine). Other manipulations were performed as described in Materials and Methods.

free methionine proceeds only via methionyl-tRNA, and it may be reasonably concluded that the methionyl-tRNA is the intermediate in the incorporation of methionine. The relatively small dependency of the incorporation on the presence of methionyl-tRNA synthetase may be ascribed to the presence of the synthetase in the supernate II. A mixture of 19 amino acids other than methionine and GTP had practically no effect on the incorporation and KCl was rather inhibitory. These results further support the fact that this incorporation is not due to the ribosomal contamination. Puromycin inhibited the incorporation. RNase A and trypsin inhibited, as expected, while DNase I did not.

The incorporation of methionine was not reduced when the mixture was further incubated with RNase A after the standard incubation (complete II in the Table II). This may indicate that the methionine is incorporated into protein. The methionyl-tRNA synthetase and methionine tRNA were successfully replaced with the mixture of aminoacyl-tRNA synthetases and the mixture of tRNA's, respectively, although a little less incorporation was the case (complete III in the Table II).

As described above, the incorporation of methionine was dependent on the presence of casein. Table III indicates the effects of the addition of some other proteins as well as the casein upon the incorporation of methionine and leucine in an incubation mixture including both methionyl- and leucyl-tRNA synthesizing systems at the saturating level. The incorporation of methionine was dependent on the presence of casein or α-casein. There was little incorporation in the presence of albumin and α-lactalbumin, but after arginylation of these proteins the activities were dramatically increased. α_{s1}-Casein (24), a major component of casein and α-casein, and the arginylated albumin (4) were shown to carry arginine residues at the amino terminals, and the α-lactalbumin having glutamic acid residue at the amino terminal (25) was possibly arginylated (14). Accordingly, the results obtained here may suggest that the methionine is incorporated

into arginine residue at the amino terminal of protein. Similar requirements were observed for the incorporation of leucine by the leucyl, phenylalanyl transferase (11).

Discussion

The data reported here demonstrate that a soluble, heat-labile and non-dialyzable factor(s) from Escherichia coli B incorporates methionine into the 95°C CCl_3COOH-insoluble fraction. The incorporation only proceeded via methionyl-tRNA. The presence of casein, arginylated albumin or arginylated α-lactalbumin was essential to the incorporation, and methionine was possibly incorporated into the protein molecules. These results may reasonably suggest the occurrence of methionyl-tRNA:protein methionyl transferase in Escherichia coli B. Hird et al. reported the incorporation of methionine into protein by the soluble system from rat liver and the incorporation appears to be insensitive to RNase A (26). The incorporation of methionine described here is different because of the sensitivity to RNase A and absolute requirement of the methionyl-tRNA synthesizing system or methionyl-tRNA. About 45% of the amino terminals of proteins in Escherichia coli B were found to be occupied by methionine residue (27). Some of these proteins might have been methionylated by the methionyl transferase post-translationally (4).

The data obtained here also suggest that methionine is incorporated into arginine residue at the amino terminal of protein. Leucine and phenylalanine have been demonstrated to be incorporated into amino terminals of proteins having arginine residues at the terminals by the well characterized enzyme, leucyl-, phenylalanyl-tRNA:protein leucyl, phenylalanyl transferase (11, 15), which has been claimed to be specific to leucyl- and phenylalanyl-tRNA (12, 28, 29). Our preliminary data (not shown here) indicated that leucyl- and phenylalanyl-tRNA are inhibitory to the incorporation of methionine. Thus, as one of the possibilities it is considered that the methionyl transferase is the same as the leucyl, phenylalanyl transferase. Before this

conclusion can be accepted further detailed studies must be made, and they are now in the process in this laboratory.

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