POLY(A)-DIRECTED FORMATION OF THIALYSINE OLIGOPEPTIDES IN E. *coli* CELL-FREE SYSTEM

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It was proved in the poly(A)-directed system that thialysine can participate in all stages of protein biosynthesis and replace the natural lysine.

Thialysine is an isosteric analogue of lysine, containing a sulfur atom in position 4 of the molecule instead of the methylene group[1]. Experiments with purified lysyl-tRNA synthetase have demonstrated that thialysine inhibits incorporation of [^{14}C]-lysine into *E. coli* tRNA (ref.[2,3]). In these experiments no radioactive thialysine was available. Stern and Mehler[4] performed an experiment with purified lysyl-tRNA synthetase and [^{35}S]-thialysine. They found an incorporation of thialysine into tRNA, however the value of K_m was for thialysine approximately by three orders higher than for lysine. Stern and Mehler also determined radioactivity in hot trichloroacetic acid insoluble material from a lysine auxotroph of *E. coli* grown in a medium, containing both lysine and [^{35}S]-thialysine. They did not, however, state whether the radioactive sulfur was contained in thialysine or in some other product. Goldberg[5] had noted that in the presence of thialysine anomalous proteins were synthesized in *E. coli* characterized by a higher rate of degradation. A similar phenomenon was observed independently also by Hermann and coworkers[6].

The above *in vivo* experiments indicate the incorporation of the analogue into protein only indirectly. In view of the various possible metabolic changes of thialysine the finding of ^{35}S-label in the hot trichloroacetic acid insoluble material does not necessarily mean the incorporation of thialysine as such into protein.

In the present study we investigated the *in vitro* incorporation of thialysine into the polypeptide chain in a poly(A)-directed system from *E. coli*. In this system natural lysine gives rise to lysine oligopeptides ranging from dilysine to heptalysine[7]. The present study was made possible by a new method of synthesis of highly labeled [^{14}C]-thialysine designed by one of the authors[8].

EXPERIMENTAL

Materials

[1,2,3-^{14}C]-L-thialysine was prepared enzymatically from [U-^{14}C]-L-serine obtained from the Institute for Research, Production and Use of Radioisotopes, Prague, and cysteamine[8]. The resulting preparation (75 μC/mol) was pure according to chromatography and high-voltage electrophoresis. [U-^{14}C]-L-lysine (75 μCi/μmol) was obtained from the Institute for Research, Production and Use of Radioisotopes, Prague. ATP and poly(A) were purachsed from Calbiochem. Transfer RNA, S-30 and S-100 supernatants were prepared from *E. coli* B, harvested during the logarithmic phase, by the methods of Littauer and coworkers[9] and Nirenberg and Matthaei[10] respectively.

Methods

Aminoacylation of tRNA was assayed by a standard method using protamine sulfate fractionated S-100 postribosomal supernatant[3] and 0·1 μC of [^{14}C]-lysine or [^{14}C]-thialysine.

Poly(A)*-directed cell-free synthesis* was performed essentially by the method of Rychlík[7]. The reaction mixture contained in 0·1 ml: 0·1M Tris-acetate (pH 7·8), 0·1M-NH_4Cl, 0·01M magnesium acetate, 1·8 mMβ-mercaptoethanol, 10 μg of poly(A), 0·18 μmol of ATP, 0·5 mg of tRNA, 0·6 mg of protein of S-30 fraction and 0·14 μC of [^{14}C]-lysine or [^{14}C]-thialysine. Incubations were stopped after 20 min at 35°C by adding 3 ml of cold 5% trichloroacetic acid. The precipitate was filtered through a Millipore filter, washed three times with 3 ml portions of cold 5% trichloroacetic acid, dried and counted. Radioactivity in the precipitate represents the sum of lysyl-tRNA and oligolysyl-tRNA. From each value radioactivity of the control, treated with hot trichloroacetic acid in which lysine as well as oligolysine peptides are soluble, was subtracted.

*Determination of peptidyl-*tRNA *and aminoacyl-*tRNA formed in the poly(A)-directed system. After incubating the 0·1 ml reaction mixture described above for 20 min at 35°C, 10 μl of 2M sodium acetate (pH 5·5) containing 0·1M-$CuSO_4$ was added and incubated for another 20 min at 35°C. The reaction was stopped by adding 3 ml of cold 5% trichloroacetic acid; further procedures were as above. The radioactivity represents oligolysyl-tRNA.

Isolation of lysine peptides on the carboxymethyl cellulose column: For the analysis of peptides synthesized in a poly(A)-directed system the reaction was carried out in a volume 0·3 ml with 0·5 μCi of labelled lysine or thialysine. [^{14}C]-oligolysyl-tRNA and/or [^{14}C]-oligothialysyl-tRNA was isolated from the reaction mixture by phenol extraction, oligopeptides were split off and separated on the carboxymethyl cellulose column by an exponential gradient of a pyridine–acetate buffer as described in ref.[11].

Radioactivity measurement: All samples were measured in a proportional gas-flow windowless Frieseke-Hoepfner counter.

RESULTS AND DISCUSSION

Poly(A) directs the formation of polylysine in the cell-free protein synthesizing system[12]. Lysine peptides are soluble in hot trichloroacetic acid and therefore one cannot distinguish between oligolysyl-tRNA and lysyl-tRNA on the basis of different solubility in hot trichloroacetic acid. However, incorporation of labeled

lysine into peptidyl-tRNA and aminoacyl-tRNA in a poly(A)-dependent system can be distinguished by selective hydrolysis of the ester bond in aminoacyl-tRNA by the catalytic effect of Cu^{2+}-ions. A free α-amino group is required for this action and, therefore, the ester bond in peptidyl-tRNA is resistant to hydrolysis. The catalytic effect of Cu^{2+}-ions for discriminating the unblocked aminoacyl-tRNA from the corresponding N-acyl derivative was described by Zamecnik[13]. In our laboratory we have applied this procedure for the quantitative determination of oligolysyl-tRNA and lysyl-tRNA (unpublished results).

Using the technique described, the poly(A)-directed incorporation of [^{14}C]-lysine and/or [^{14}C]-thialysine into peptidyl-tRNA and aminoacyl-tRNA was followed. Table I shows that under identical conditions the analogue is incorporated into peptidyl-tRNA and that the incorporation is by one order of magnitude lower than that of the natural lysine.

The same difference was found also in the direct aminoacylation of tRNA by lysine and thialysine. In this case, too, given the same conditions, about 10-fold more lysyl--tRNA than thialysyl-tRNA was formed (Fig. 1). This finding corresponds to the

TABLE I

Poly(A)-Directed Incorporation of ^{14}C-Lysine and ^{14}C-Thialysine

The results are given in pmol of the amino acid incorporated into peptidyl-tRNA or aminoacyl-tRNA. Details of reaction conditions are given under Methods.

Amino acid	Peptidyl-tRNA	Aminoacyl-tRNA
[^{14}C]-Lysine	542	58
[^{14}C]-Thialysine	56	4·5

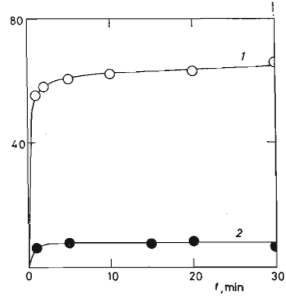

FIG. 1

Time Course of Lysine and Thialysine Incorporation into tRNA

1 [^{14}C]-lysine, *2* [^{14}C]-thialysine Incorporation is expressed in pmol per 0·1 mg tRNA. For details see Experimental.

earlier observation of Stern and Mehler[4] concerning the reduced affinity of thialysine to lysyl-tRNA synthetase.

In order to characterize precisely the synthesized product we isolated the intermediate of the reaction, *i.e.* peptidyl-tRNA. The poly(A)-dependent system is particularly suitable for this purpose[7]. The lysine peptides split off from tRNA were analyzed on a carboxymethyl cellulose column by an exponential gradient of pyridine–acetate (pH 5·2) (ref.[11]). Under the conditions employed all free amino groups in lysine as well as thialysine peptides are fully ionized and the elution takes place according to the increasing positive charge, *i.e.* first is eluted dilysine, then trilysine *etc.* (Fig. 2, curve *1*). The elution profile of the peptides split off from [^{14}C]-oligothialysyl-tRNA (curve 2) agrees with that of lysine peptides.

The low incorporation of thialysine into oligopeptides is apparently due to the reduced supply of thialysyl-tRNA. It appears that the subsequent reactions, *i.e.* binding of thialysyl-tRNA to ribosome, transpeptidation and translocation proceed unimpeded. This conclusion is supported by the similarity of distribution of lysine and thialysine peptides (Fig. 2) and by the data (Table I) showing that thialysyl-tRNA, like lysyl-tRNA, does not accumulate as it should if the subsequent proteosynthetic reactions were blocked by the use of the lysine analogue.

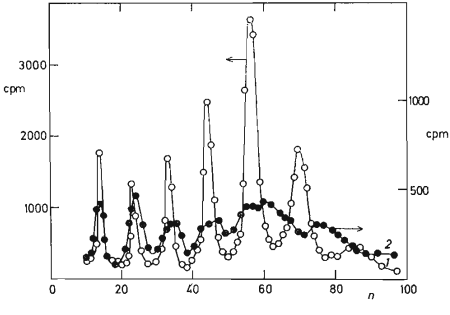

FIG. 2

Products of the *in vitro* Poly(A)-Directed Synthesis

Separation on the carboxymethyl cellulose column[11]: curve *1* lysine peptides, curve 2 thialysine peptides. Peaks from the left correspond to di-, tri-, tetrapeptide *etc.*

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