THE INITIATION OF PEPTIDE CHAIN SYNTHESIS IN SYSTEMS DIRECTED BY MESSENGER WITH NO INITIATOR CODON

M. FÁBRY and I. RYCHLÍK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received July 7th, 1973

The initiation of peptide chain synthesis in systems in which the messenger contained no initiator codon was studied using the poly(A)-directed system of *Escherichia coli*. With the dinitrophenylation technique it was found that the nascent lysine peptides had a free N-terminal amino group and that thus neither the blocked lysyl-tRNA nor the preformed peptidyl-tRNA participated in the initiation of oligolysine synthesis, nor did a misreading of N-formylmethionyl-tRNA for lysyl-tRNA take place. Apparently the first peptide bond is formed by the transfer of the lysine residue from the lysyl-tRNA to the adjacent molecule of lysyl-tRNA.

The mechanism underlying the initiation of polypeptide chain synthesis in the cell-free system of *Escherichia coli* involves under normal conditions the participation of an initiator tRNA ($fMet\text{-}tRNA_F$)* responding to initiator codons AUG or GUG, several initiation factors, ribosomes, and GTP (ref.[1]). At a higher concentration of Mg^{2+}-ions, however, the formation of peptides can be directed also by synthetic polyribonucleotides containing no initiator codon; thus, *e.g.* polylysine is produced in the poly(A)-directed system[2]. In such cases the mechanism responsible for the start of synthesis is not entirely clear.

In a poly(A)-directed cell-free system from *E. coli* several possibilities of initiation can be assumed: *1*) At a higher concentration of Mg^{2+}ions, which in the poly(A) system is essential for initiation, lysyl-tRNA though having a free $\alpha\text{-}NH_2$-group enters the donor ribosomal site and hence can start the synthesis. *2*) At a higher concentration of Mg^{2+} ions mRNA is misread, AUG being read instead of triplet AAA, and initiation takes place according to the general pattern utilizing $fMet\text{-}tRNA_F$ (*cf.*[3]). *3*) In the S-30 fraction peptidyl-tRNA remains attached to the ribosomal donor site from which, after the addition of poly(A), the preformed peptide is transferred to the $\alpha\text{-}NH_2$-group of lysine in lysyl-tRNA bound to the acceptor site. Polylysine synthesis then continues on this preformed peptide (*cf.*[4,5]).

In the first case the produced lysine peptides will possess a free $\alpha\text{-}NH_2$-group, while in the second and third case this group will not be free. Thus the finding whether

* Abbreviations used: $fMet\text{-}tRNA_F$ N-formylmethionyl-$tRNA_F$, ε-DNP-lysine N^{ε}-(2,4-dinitrophenyl)-L-lysine, di-DNP-lysine $N^{\alpha}N^{\varepsilon}$-bis(2,4-dinitrophenyl)-L-lysine.

the α-NH_2-group of oligolysine synthetized in the poly(A)-directed system is free or blocked, might throw light on the mode of initiation in this system.

EXPERIMENTAL

Materials. ATP was obtained from Koch. Light & Co., Ltd., Colnbrook (England), polyadenylic acid from Calbiochem (U.S.A.), L-lysine-[^{14}C] (spec. act. 85 mCi/mmol) from the Institute for Research, Production and Uses of Radioisotopes, Prague, ε-DNP-lysine and di-DNP-lysine were products of the Mann Research Laboratories (U.S.A.). Nonradioactive lysine peptides were obtained by partial hydrolysis of cold polylysine (donated by Dr K. Bláha of this Institute) and separated by paper chromatography according to Walley and Watson[6]. The S-30 fraction was prepared according to Nirenberg and Matthaei[7] and [^{14}C]-oligolysyl-tRNA according to Rychlík[8].

Lysine peptides were split off from oligolysyl-tRNA, separated and isolated on the carboxymethyl cellulose column with an exponential gradient of a pyridine—acetate buffer as described in ref.[9].

Dinitrophenylation of lysine peptides. To the radioactive peptide (isolated on the column of CM-cellulose) 200 nmoles of nonradioactive peptide of the same number of lysine residues were added as a carrier and dried. Then 0·06 ml of 1% trimethylamine and 0·12 ml of ethanolic solution of 2,4-dinitro-1-fluorobenzene (5 mg/ml) were added. After 2 h incubation at 40°C the same amount of trimethylamine and 2,4-dinitro-1-fluorobenzene was added again, and incubated for another 2 h. During the reaction the pH value was maintained above 8·0 by adding trimethylamine. The excess of the dinitrophenylating agent was converted into 2,4-dinitroaniline by incu-

TABLE I

The Proportion of Free α-Amino Groups in Lysine Peptides Formed in a Poly(A)-directed System

Peptide	Experiment	Molar ratio ε-DNP-lysine/di-DNP-lysine			
		measured by		after correction to the internal standard[a]	theoretical value
		spectrophotometry	radioactivity		
Dilysine	1	1·16	1·02	0·88	1·0
	2	0·89	0·85	0·96	1·0
Trilysine	1	2·15	2·30	2·14	2·0
	2	1·91	1·93	2·02	2·0
Tetralysine	1	2·68	2·68	3·00	3·0
	2	2·78	2·69	2·90	3·0

[a] The values in column 3 represent the corrected ratios of radioactivity between ε-DNP-lysine and di-DNP-lysine in the given peptides. They were obtained by correcting the measured radioactivity ratios (column 2) according to the internal standards (column 1). The resultant values agree well with the theoretical ratios of the standards added. For further details see Experimental.

bation for 1 h with 10 μl of 25% NH_3 at 40°C. After completion of the reaction the mixture was evaporated to dryness, dissolved in a small volume of dimethylformamide and applied to Whatman DE 81 paper which, prior to use, was washed in 50% acetic acid and dried. Following 2 h equilibration the DNP-peptide was separated from 2,4-dinitrophenol and 2,4-dinitroaniline (which were formed in the course of reaction) by descending chromatography (0·1M pyridine–acetate buffer pH 6·2 containing 20% of acetone v/v; 1—2 h) and the pure DNP-peptide was eluted from the origin of the chromatogram with 80% acetic acid–acetone 10 : 3, v/v (Fig. 1).

After drying the DNP-peptide was dissolved in the smallest possible amount of glacial acetic acid, 0·3 ml of 6N-HCl was added, the tube was flushed with N_2, sealed and the content hydrolyzed for 16 h at 105—110°C.

The hydrolysate from which HCl was removed by repeated drying was dissolved in a small volume of dimethylformamide and applied to Whatman DE 81 paper and equilibrated overnight. The mixture was then separated by descending chromatography (0·1M pyridine–acetate pH 6·2 containing 20% of acetone v/v; 6 h). di-DNP-Lysine, 2,4-dinitrophenol and traces of the incompletely hydrolyzed DNP-peptide were left at the origin of the chromatogram whereas ε-DNP-lysine moved up to half of the length of the paper. Free lysine-[^{14}C] which could result from the incomplete reaction of the peptide with 2,4-dinitro-1-fluorobenzene moves ahead of ε-DNP-lysine and thus cannot distort the value of radioactivity measured in ε-DNP-lysine (Fig. 2*a*).

After drying, the lower part of the paper containing ε-DNP-lysine was cut off and in the upper

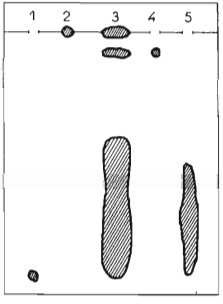

FIG. 1

Isolation of the DNP-Peptide

Descending chromatography on Whatman DE 81 paper (0·1M pyridine-acetate buffer pH 6·2 containing 20% of acetone v/v; 1—2 h). 1 Standard of ε-DNP-lysine, 2 standard of di-DNP-lysine, 3 DNP-peptide, 4 2,4-dinitropehnol, 5 2,4-dinitroaniline.

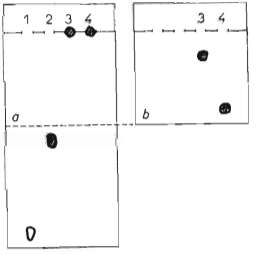

FIG. 2

Separation of ε-DNP-Lysine and di-DNP-Lysine

Descending chromatography on Whatman DE 81 paper with the successive use of 0·1M pyridine–acetate buffer pH 6·2 containing 20% of acetone v/v, 6 h (*a*) and 50% acetic acid, 30 min (*b*). 1 Lysine, 2 ε-DNP-lysine, 3 di-DNP-lysine, 4 2,4-dinitrophenol.

part di-DNP-lysine was separated from 2,4-dinitrophenol by descending chromatography (50% acetic acid; 0·5 h) (Fig. 2*b*).

The yellow spots of ε-DNP-lysine and di-DNP-lysine were cut out, eluted with 80% acetic acid and the extinction of the samples was measured at 360 nm. The extinction value for di-DNP-lysine was divided by the factor 1·8 derived from the ratio of molar extinction coefficients of di-DNP-lysine and ε-DNP-lysine in 80% acetic acid. Following the measurement of extinction samples were evaporated on stainless planchetes and their radioactivity measured in a proportional gas-flow windowless Frieseke-Hoepfner counter.

In order to correct all possible errors of the method (incomplete reaction of the peptide with the agent, incomplete hydrolysis or perhaps the conversion of di-DNP-lysine to ε-DNP-lysine during hydrolysis, losses at the isolation of individual DNP-derivatives, *etc.*) a nonradioactive peptide of the same number of lysine residues was added as a carrier to the studied radioactive peptide. For example, to dilysine-[^{14}C] an excess of standard dilysine was added, from which at an ideal course of reaction and hydrolysis both derivatives, ε-DNP-lysine and di-DNP-lysine, must be obtained in a 1 : 1 ratio. The actual ratio of both derivatives was determined by spectrophotometry. This ratio differed more or less from the theoretical value due to the above-mentioned methodical errors and, therefore, the ascertained ratio of radioactivity of ε-DNP-lysine to di-DNP-lysine was adequately corrected.

RESULTS AND DISCUSSION

The poly(A) system employed permits an easy isolation of oligolysyl-tRNA, the direct intermediate of proteosynthesis. The typical distribution of lysine peptides (after splitting from tRNA) on the carboxymethyl cellulose column is shown in

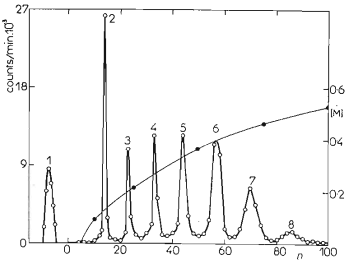

FIG. 3

Synthesis of Lysine Peptides in the Poly(A)-Directed System of E. *coli*

Chromatographic separation of lysine peptides split off from oligolysyl-tRNA was performed on carboxymethyl cellulose with an exponential gradient of pyridine–acetate buffer pH 5·2 (0·005—0·54M). Radioactivity of fractions (o), buffer molarity (●). **1** Washings, **2** dilysine, **3** tri-*l*ysine, **4** tetralysine, **5** pentalysine, **6** hexalysine, **7** heptalysine, **8** octalysine.

Fig. 3. These peptides represent approx. 90% of all radioactivity contained in the peptidyl-tRNA. The remaining 10% of radioactivity are not adsorbed to the column and are presented in the figure as washings. Half of this fraction represents free lysine (di-DNP-lysine is formed primarily by dinitrophenylation) and the other half (*i.e.* 5% of the total radioactivity) contains non-hydrolyzed peptidyl-tRNA.

A similar distribution of lysine peptides attached to tRNA was observed by Bretscher[10], Smith[11] and Rychlík[8]. These authors identified the individual peaks of lysine radioactivity by cochromatography with synthetic lysine peptides at pH 3 – 5. Although considered as sufficient proof for identification, this technique would not resolve adequately α-NH_2-blocked lysine peptides and unblocked ones, the latter being shorter by a lysine residue (*i.e.* blocked trilysine from unblocked dilysine). To overcome this drawback a more direct approach was used, comparing the proportion of N-terminal lysine and internal lysine in the peptide by DNP-technique.

All lysine peptides which were analyzed (peaks 2, 3 and 4 in Fig. 3) contained at the N-terminal position lysine with a free α-NH_2-group. After complete dinitrophenylation of the peptides, hydrolysis and chromatography we obtained besides ε-DNP-lysine, which is produced from the lysine localized in the inner position of the peptide, also di-DNP-lysine which can be formed only from the N-terminal lysine having a free α-NH_2-group (Table I). If the α-NH_2-group of lysine were blocked di-DNP-lysine could not be formed.

In view of the fact that the peptides are homogenously labelled with ^{14}C it is possible to calculate the content of unblocked N-terminal lysine in the peptide from the ratio of radioactivity between ε-DNP-lysine and di-DNP-lysine. In the case of dilysine the ε-DNP-lysine : di-DNP-lysine ratio should be 1 : 1, in case of trilysine 2 : 1 *etc.* The ratio of radioactivity ε-DNP-lysine : di-DNP-lysine after the correction to the internal standard shows that the measured values correspond to the theoretical values anticipated for unblocked dilysine, trilysine and tetralysine. Since also in the first intermediary product of synthesis, in lysyllysyl-tRNA, the terminal lysine has a free α-NH_2-group, it follows that the synthesis of peptides in the poly(A)-directed system starts with the formation of dipeptidyl-tRNA from two aminoacyl-tRNAs. The same holds probably also for other systems directed by mRNAs possessing no initiator codons.

The other mechanisms of initiation discussed here, *i.e.* initiation by means of preformed peptidyl-tRNA, lysyl-tRNA with blocked α-NH_2-group of lysine or by means of miscoded fMet-$tRNA_F$, provided these work at all, can play only a negligible part in the poly(A)-directed system.

On the other hand, as was found by Castles and coworkers[4], Travis and coworkers[5] and van der Zeijst and coworkers[12] in cell-free systems derived from eukaryotic cells with poly (U) messenger, phenlyalanine residues are added to endogenous peptidyl-tRNA preformed on ribosomes, *i.e.* the mechanism 3 plays the principal role.

REFERENCES

1. Lucas-Lenard J., Lipmann F.: Ann. Rev. Biochem. *40*, 409 (1971).
2. Gardner R. S., Wahba A. J., Basilio C., Miller R. S., Lengyel P., Speyer J. F.: Proc. Natl. Acad. Sci. US *48*, 2087 (1962).
3. Matthaei H., Love K., Milberg M., Sander G., Swan D., Voigt H. P. in the book: *Molecular Genetics* (H. G. Wittmann, H. Schuster, Eds), p. 81. Springer, Berlin—Heidelberg—New York 1968.
4. Castles J.J., Rolleston F. S., Wool I. G.: J. Biol. Chem. *246*, 1799 (1971).
5. Travis R. L., Lin C. Y., Key J. L.: Biochim. Biophys. Acta *277*, 606 (1972).
6. Walley S. G., Watson J.: Biochem. J. *55*, 328 (1953).
7. Nirenberg M. W., Matthaei J. H.: Proc. Natl. Acad. Sci. US *47*, 1588 (1961).
8. Rychlík I.: This Journal *30*, 2259 (1965).
9. Pulkrábek P., Rychlík I.: Biochim. Biophys. Acta *155*, 219 (1968).
10. Bretscher M. S.: J. Mol. Biol. *12*, 913 (1965).
11. Smith J. D.: J. Mol. Biol. *8*, 772 (1964).
12. Van der Zeijst B. A. M., Engel K. J., Bloemers H. P. J.: Biochim. Biophys. Acta *294*, 517 (1973).