ON SUBSTRATE SPECIFICITY OF THE DONOR SITE OF THE *ESCHERICHIA COLI* RIBOSOMAL PEPTIDYL TRANSFERASE CENTER

Synthesis of dipeptides from 3'-terminal fragments of aminoacyl-tRNA

S. BOURD, L. VICTOROVA and M. KUKHANOVA
Institute of Molecular Biology, USSR Academy of Sciences, Vavilov str. 32, Moscow 334, 117 984, USSR

Received 22 March 1982

1. Introduction

In the ribosome aminoacyl-tRNA and peptidyl-tRNA interact with the A-site and the P-site, respectively. One of the indications of discrimination of substrates in the peptidyl transferase center (PTC) of ribosomes is the presence of a free NH_2-group in the amino acid residue; the compounds with the protected NH_2-group bind to the P-site and serve as peptide donors whereas those with a free amino group bind to the A-site. Having studied substrate specificity of the A-site, we report here that, besides the A-site, 3'-terminal fragments of aminoacyl-tRNA, CCA-Phe, CACCA-Phe, CACCA-Leu, CACCA-Val and UCAUCACCCACCA-Val, bind effectively to the P-site of the ribosome thereby forming appropriate dipeptidyl oligonucleotides. A comparison is also made of donor activities of CACCA-Phe with CACCA-Phe←Ac and pA-Met←f.

2. Materials and methods

2.1. *Preparation of ribosomes and substrates*

The ribosomes (tight couples) from *E. coli* MRE-600 were isolated in a sucrose density gradient in a zonal rotor [1] and activated before the following experiment [2]; pA-Met←f was synthesized by the imidazole method [3]. For aminoacylation, a total preparation of tRNA from *E. coli* B was used. Fragments CACCA-[^{14}C]Phe, CACCA-[^{14}C]Leu, CACCA-[^{14}C]Val and UCAUCACCCACCA-[^{14}C]Val were obtained by T_1-ribonuclease hydrolysis of appropriate aminoacyl-tRNA and subsequent purification by high-voltage electrophoresis on the Whatman no. 3 MM paper with the solvent system pyridine–acetic acid buffer (0.5%:5%) (pH 3.5) at 53 V/cm for 2.5 h [4]. Hydrolysis of CACCA-[^{14}C]Phe with T_2-ribonuclease afforded CCA-[^{14}C]Phe which was then isolated by electrophoresis in similar conditions. Acetylation of isolated aminoacylated products was done with acetic anhydride. The resulting material was purified with electrophoresis. [^{14}C]Valine had spec. act. 175 mCi/mmol (ÜVVVR, Czechoslovakia), [^{14}C]phenylalanine, 522 mCi/mmol, [^{14}C]leucine, 354 mCi/mmol (Amersham).

2.2. *Transfer assay and identification of the reaction products*

The reaction was run in a 50 μl mixture containing 50 mM Tris–HCl (pH 7.4), 20 mM $MgCl_2$, 200 mM KCl, a 40% ethanol (v/v) at 0°C. The amounts of ribosomes and aminoacyl oligonucleotides and the reaction times are given in the figure legends. The reaction was terminated by adding lincomycin to 1 mM final conc. The samples were centrifuged for 15 min at 9000 × *g*, dried in vacuo. Then hydrolysis was performed with 0.5 N NaOH for 1 h at 37°C, and the mixture applied to Whatman no. 1 paper

The reaction products were identified by chromatography in systems: *n*-BuOH:water:AcOH, 78:17:5 (system A); *n*-BuOH (saturated with a 25% solution of NH_4OH) and ethylacetate (4:1, v/v) (system B) or by electrophoresis under the above conditions. Chromatograms and electrophoregrams were cut into 1 cm bands, and radioactivity assayed in 10 ml toluene scintillator PPO–POPOP in a SL-40 (Intertechnique, France) counter. In system A, R_F = 0.33 for valine

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

and R_F = 0.67 for divaline; in system B R_F = 0.44 for valine, R_F = 0.72 for divaline. Disposition of peaks for phenylalanine and diphenylalanine at electrophoresis was 0.27.

3. Results

3.1. *Formation of dipeptidyl oligonucleotides upon interaction of aminoacylated fragments with ribosomes in fragment reaction conditions*

The reaction products after incubation of the model acceptors CACCA-[^{14}C]Phe, CACCA-[^{14}C]Val or UCAUCACCCACCA-[^{14}C]Val with ribosomes in a 40% ethanol and subsequent hydrolysis were separated by high-voltage electrophoresis or paper chromatography. Identification of reaction products was done by comparison to chemically synthesized markers.

In chosen conditions the incubation of aminoacylated fragments with ribosomes led to a rather intense formation of dipeptidyl oligonucleotides (table 1). After hydrolysis of the reaction products, dipeptides were found even in the case when the ratio (ribosomes:aminoacyl pentanucleotides) exceeded 3:1. Synthesis of dipeptides was inhibited by the antibiotic lincomycin and by tRNA (table 1) and ceased to continue in the absence of alcohol, which pointed to the ribosomal nature of this reaction. Table 1 shows that incubation of CCA-Phe with ribosomes also resulted in formation of CCA-PhePhe (after hydrolysis, diphenylalanine was identified) with effectiveness close to the case of CACCA-Phe.

Fig.1 illustrates the rate of formation of CACCA-PhePhe upon incubation of CACCA-Phe with ribosomes; 50% of the product formed arises in the first few minutes of incubation.

Table 1
Formation of dipeptides upon incubation of aminoacyl oligonucleotides with ribosomes in the presence of a 40% ethanol

No. exp.	Reaction assay				Reaction products after hydrolysis[a]		
	Ribosomes (pmol)	Substrate (pmol)	[substrates] / [ribosomes]	Additions	Dipeptide (pmol)	Amino acid (pmol)	[dipeptide] / [amino acid]
1	33.8	CACCA-Phe					
		9.8	0.29	–	1.0	2.4	0.44
		19.6	0.58	–	2.3	3.5	0.66
		39.2	1.16	–	5.3	7.3	0.72
2	67.5	CACCA-Val					
		14.0	0.2	–	2.3	7.5	0.30
		73.0	1.1	–	12.1	24.6	0.49
		146.0	2.2	–	24.7	49.4	0.50
		146.0	2.2	Lincomycin			
		146.0	2.2	(1 mM)	4.5	81.8	0.06
		146.0	2.2	tRNA (9 μM)	7.4	91.3	0.08
3	56.0	CACCA-Leu					
		156.0	2.78	–	18.9	79.0	0.24
4	195.0	CCA-Phe					
		54.6	0.28	–	5.6	19.4	0.29
		72.2	0.37	–	7.9	26.3	0.30
		109.2	0.56	–	11.9	40.1	0.30
		UCAUCACC					
5	62.0	CACCA-Val					
		295.0	4.75	–	14.6	81.7	0.18

[a] Radioactivity found on electrophoregrams was 50–60% from the amount introduced into the sample. The loss was, obviously, connected with smaller effectiveness of counting on paper and treatment of samples

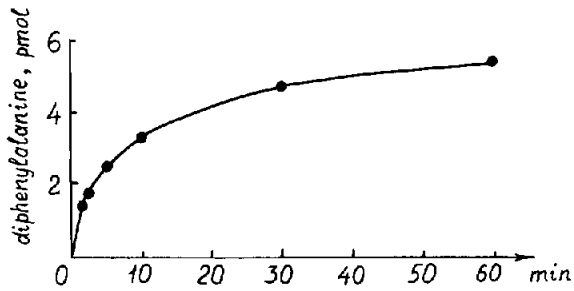

Fig.1. Formation of [^{14}C]Phe[^{14}C]Phe *vs* time. The reaction mixture contained 64 pmol CACCA-[^{14}C]Phe and 67 pmol ribosomes.

3.2. *Demonstration of binding of CACCA-Phe to the PTC donor site*

Model donors of peptide pA-Met←f and CACCA-[^{14}C]Phe←Ac inhibited formation of CACCA-PhePhe. At $\geqslant 6.4 \times 10^{-4}$ M pA-Met←f, a 2-fold inhibition of diphenylalanine formation within 10 min takes place, and within 60 min, a 4–5-fold inhibition (table 2). Fig.2 shows that fMetPhe was identified as the major product of hydrolysis. Utilization of CACCA-[^{14}C]-Phe as the acceptor in this system reached 45–50%.

If CACCA-[^{14}C]Phe←Ac was introduced into the reaction instead of Pa-Met←f, in the incubation material there were found phenylalanine, diphenylalanine, acetylphenylalanine and acetyldiphenylalanine. Separation of all these reaction products was achieved by electrophoresis (fig.3). Fig.4 illustrates the ratio of condensation products diphenylalanine and acetyldiphenylalanine upon raising CACCA-[^{14}C]Phe←Ac to

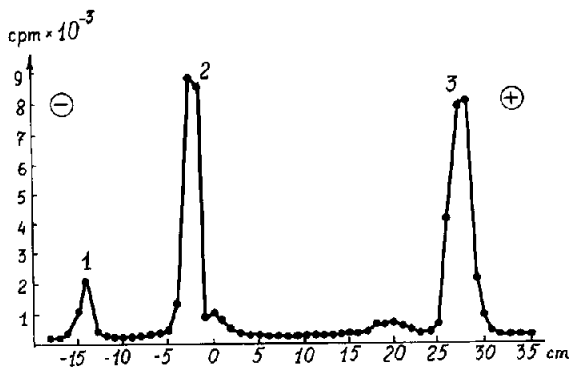

Fig.2. Electrophoregram of reaction products CACCA-[^{14}C]-Phe and pA-Met←f. The sample contained 64 pmol CACCA-[^{14}C]Phe, 67 pmol ribosomes and 1.6 mM pA-Met←f. Time of the reaction was 1 h: (1) [^{14}C]Phe[^{14}C]Phe; (2) [^{14}C]Phe; (3) fMet-[^{14}C]Phe.

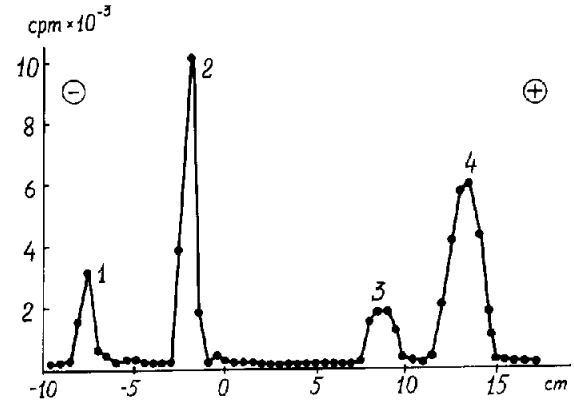

Fig.3. Electrophoregram of reaction products CACCA-[^{14}C]-Phe and CACCA-[^{14}C]Phe←Ac. The sample contained 35.2 pmol CACCA-[^{14}C]Phe, 45.5 pmol CACCA-[^{14}C]Phe←Ac and 52.5 pmol ribosomes: (1) [^{14}C]Phe[^{14}C]Phe; (2) [^{14}C]-Phe; (3) Ac-[^{14}C]Phe[^{14}C]Phe; (4) Ac-[^{14}C]Phe.

CACCA-[^{14}C]Phe, the amount of diphenylalanine formed was decreased whereas that of acetylphenylalanine increased, which can be explained by their competition for the P-site.

3.3. *Effectivity of dipeptidyl oligonucleotide formation*

Fig.5 summarizes the data on the formation rate of diphenylalanine and acetyldiphenylalanine in the reaction mixture into which 25.4 pmol CACCA-[^{14}C]-Phe and 37.8 pmol CACCA-[^{14}C]Phe←Ac were introduced. The formation rate of diphenylalanine was close to that of acetyldiphenylalanine (fig.5). The

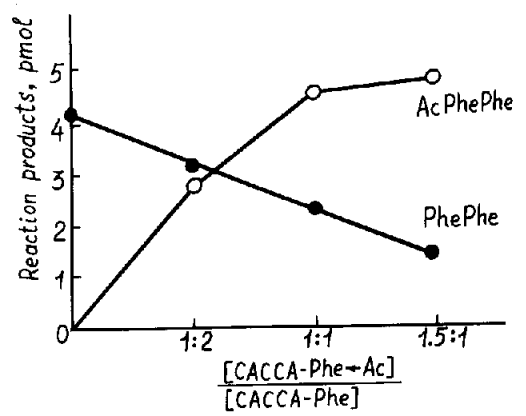

Fig.4. Formation of PhePhe and AcPhePhe in the presence of the rising amount of CACCA-Phe←Ac. The sample contained 35.3 pmol CACCA-[^{14}C]Phe and 67 pmol ribosomes.

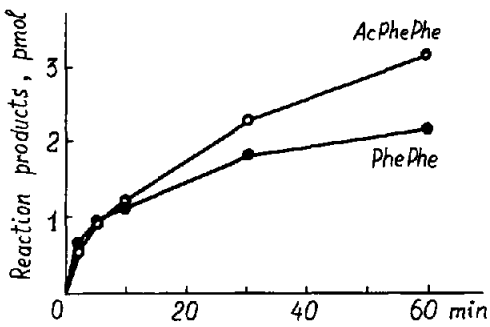

Fig.5. Formation of Ac-[^{14}C]Phe[^{14}C]Phe and [^{14}C]Phe-[^{14}C]Phe *vs* time. The sample contained 25.4 pmol CACCA-[^{14}C]Phe, 37.8 pmol CACCA-[^{14}C]Phe←Ac and 67 pmol ribosomes.

total utilization of acceptor for these two reactions was only 20%, which is considerably lower as compared to when pA-Met←f was reacted with CACCA-[^{14}C]Phe (table 2) or CACCA-[^{14}C]Phe←Ac with puromycin [5].

4. Discussion

The above experiments showed that aminoacyl oligonucleotide fragments of aminoacyl-tRNA CACCA-Phe, CACCA-Val, UCAUCACCCACCA-Val and even CCA-Phe were effectively bound not only to the PTC A-site but to the P-site as well, which resulted in appearance of dipeptidyl oligonucleotides in the reaction mixture.

Fig.4 shows that substrates CACCA-Phe and model donor CACCA-Phe←Ac competed with one another, a 50% inhibition of formation of diphenylalanine being observed at the 1:1 ratio of substrates in the reaction mixture. This fact speaks of closeness of binding constants of both substrates to the P-site. Formation of dileucine upon UACCA-Leu binding to 50 S subunits in 50% ethanol in amounts up to 10% from the bound fragment was reported in [6]. These amounts of dipeptides were not negligible as the concentration of 50 S subunits was 10^3 greater than that of UACCA-Leu. From these results it cannot be stated that aminoacyl oligonucleotides can be bound only to the A-site.

In [7], formation of phenylalanylpuromycin occurred after incubation of CACCA-Phe with ribosomes and subsequent addition of puromycin, which also proves the localization of CACCA-Phe at the P-site. Our results demonstrate that all the tested model acceptors of the PTC can bind to the donor site. For CACCA-Leu←Ac we showed its preferential binding to the donor site, its association constant with the A-site being 1–1.5 orders of magnitude smaller in comparison with its binding constant to the P-site [8]. At the same time, for CACCA-Phe, as can be judged from the data obtained, strict selectivity of the sites was absent. Moreover, the formation of diphenylalanine was observed at different substrate: ribosomes ratios (from 0.3–2). Formation of a rather large amount of dipeptides at the ratio of CACCA-[^{14}C]Phe to ribosomes <1 can be explained by a positive cooperative effect between substrates at the PTC. In [9] we reported the absence of a cooperative effect upon binding of model substrates CACCA(3'NH)-Phe←Ac at the P-site and CACCA-Phe at A-site. This fact, however, does not exclude the existence of such an effect for the case when both substrates of the PTC were occupied with CACCA-Phe, which is now being studied.

Table 2
Formation of diphenylalanine in the presence of pA-Met←f

Reaction (min)	pA-Met←f (mM)	Reaction products after hydrolysis			
		Diphenyl-alanine (pmol)	Phenyl-alanine (pmol)	Formylmethionyl-phenylalanine (pmol)	Diphenyl-alanine (%)
10	–	4.6	52.0	–	100
	0.64	2.3	45.0	15.8	50
	1.60	2.0	47.0	13.0	43
60	–	8.0	43.0	–	100
	0.64	2.5	29.0	28.0	31
	1.60	1.85	25.0	27.0	23

Acknowledgements

The authors are thankful to Drs A. Krayevsky and B. Gottikh for their interest and support of this work, to Dr S. Streltsov for valuable discussions, to Dr S. Zavgorodny for a gift of the markers. We are much obliged to Mrs G. Moussinova for translation of the manuscript.

References

[1] Semenkov, Yu., Makhno, V. and Kirillov, S. (1976) Molekul. Biol. 10, 754–763.
[2] Miskin, R., Zamir, A. and Elson, D. (1968) Biochem. Biophys. Res. Commun. 33, 551–558.
[3] Azhayev, A. V., Popovkina, S. V., Tarussova, N. B., Florentiev, V. L., Krayevsky, A. A., Kukhanova, M. K. and Gottikh, B. P. (1977) Nucleic Acids Res. 4, 2223–2234.
[4] Pestka, S., Hishizawa, T. and Lessard, J. L. (1970) J. Biol. Chem. 245, 6208–6219.
[5] Černa, J., Rychlik, I., Žemlička, J. and Chladek, S. (1970) Biochim. Biophys. Acta 204, 203–209.
[6] Celma, M. L., Monro, R. E. and Vazques, D. (1971) FEBS Lett. 13, 247–251.
[7] Yukioka, M. and Morisawa, S. (1971) Biochim. Biophys. Acta 254, 304–309.
[8] Kukhanova, M., Streltsov, S., Victorova, L., Azhayev, A., Gottikh, B. and Krayevsky, A. (1979) FEBS Lett. 102, 198–202.
[9] Kukhanova, M., Victorova, L., Bourd, S., Gottikh, B., Krayevsky, A. and Sprinzl, M. (1980) FEBS Lett. 118, 176–179.