PEPTIDYL TRANSFERASE CENTER OF *ESCHERICHIA COLI* RIBOSOMES: INTERRELATIONS BETWEEN THE SUBSTRATES

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1. Introduction

In 1975 it was shown that cytidine-5′-phosphate stimulates the *E. coli* ribosomal reaction of pA-Met←f with Phe-tRNA^{Phe} or C-A-C-C-A-Phe [1]. The stimulating effect of cytidine 5′-phosphate is explained by the tendency of this nucleotide to be bound to the area of the donor site of the peptidyl transferase centre occupied in an ordinary process by the penultimate 3′-terminal nucleotide of peptidyl-tRNA [2–4]. By an identical mechanism cytidine 5′-phosphate also stimulates the reactions catalyzed by 50 S subunits of *E. coli* ribosomes [5] and by rat liver ribosomes [6]. All these facts indicate some allosteric effects taking place in the peptidyl transferase centre as a result of the occupancy of the ribosomal A- and P-site.

Several experiments have been reported which indicate that the activity of one ribosomal site can be influenced by the reaction taking place in the other site of the ribosomes (reviewed [7]). Among them the dependence of activity of puromycin [8] and other model acceptors [9] upon the nature of peptide donor should be mentioned. The peptidyl-tRNA bound to the donor site stimulates binding of the complex aminoacyl-tRNA + EF-I + GTP to rabbit reticulocyte ribosomes [10]. The stimulating action is displayed if a peptide residue is located at the 3′-hydroxyl, but not at the 2′-hydroxyl of the terminal adenosine of tRNA. The stimulation of acceptor pentanucleotide substrate binding to 50 S subunits of *E. coli* ribosomes by tRNA and C-C-A trinucleotide was also observed [11].

We report here on the systematic investigation of the interrelation between the model substrates of the peptidyl transferase center of *E. coli* ribosomes under the 'fragment reaction' conditions. We can show that the presence of model substrate-like inhibitor on one of the sites does not change the equilibrium association constant (K_a) of the second model substrate binding to the neighbouring site and practically does not increase the fraction of ribosomes taking part in substrate binding. However, the catalytic rate constants (k_{cat}) of the reactions of C-A-C-C-A-Phe with pA-Met←f, pA-Leu←f or pA-Phe←f are increased by 1.5 orders of magnitude in the presence of cytidine 5′-phosphate.

Abbreviations: C-A-C-C-A-Phe and C-A-C-C-A-Val, 3′-terminal fragments of Phe-tRNA^{Phe} and Val-tRNA^{Val} respectively; C-A-C-C-A-(3′-NH-Phe←Ac), 3′-terminal fragment of Ac-Phe-tRNA^{Phe}-C-C-A(3′-NH$_2$), pAa-Met←f, pA-Leu←f and pA-Phe←f, 2′(3′)-*O*-(*N*-formyl)aminoacyl derivatives of adenosine 5′-phosphate; pA(3′NH-Met←f), 3′-*N*-(*N*-formyl)-L-methyionyl-3′-deoxy-3′-aminoadenosine 5′-phosphate; pC, cytidine-5′-phosphate

2. Materials and methods

2.1. *Materials*

The ribosomes were isolated from *Escherichia coli* MRE-600 by centrifugation in a sucrose gradient in a zonal rotor, according to [12] and activated prior to the experiment by heating at 40°C for 10 min. C-A-C-C-A[^{3}H]Phe, C-A-C-C-A-[^{14}C]Phe and C-A-C-C-A-[^{14}C]Val were obtained by T_1 RNase hydrolysis of corresponding aminoacyl-tRNAs. Specific radioactivity for [^{14}]phenylalanine was 270 mCi/mmol

(UVVVR, Czechoslovakia), for [^{3}H]phenylalanine 6 Ci/mmol (Isotop, USSR) and for [^{14}C]valine 124 mCi/mmol (UVVVR). C-A-C-C-A(3'NH-[^{14}C]-Phe←Ac) was obtained from tRNA^{Phe}-C-C-A(3'NH-[^{14}C]Phe) [13] by acetylation with acetanhydride and subsequent hydrolysis with T_1 RNase. Specific radioactivity for [^{14}C]phenylalanine was 513 mCi/mmol (Amersham, England). Aminoacyl and acetylaminoacyl oligonucleotides were purified by electrophoresis on Whatman no. 1 or 3MM paper with 0.5% pyridine and 5% acetic acid (pH 3.5) buffer, for 2 h with a gradient of 53 V/cm. The yield of acetylation was tested prior to use as in [14]. pA-Met←f, pA-Phe←f, pA-Leu←f and pA(3'NH-Met←f) were synthesized according to [15] and [16], respectively.

2.2. *Methods*

The binding of C-A-C-C-A(3'-NH-[^{14}C]Phe-Ac), C-A-C-C-A-[^{3}H]Phe and C-A-C-C-A-[^{14}C]Val to ribosomes was examined according to [14]. The 0.05 ml incubation mixture contained 0.06 M Tris–HCl buffer (pH 7.5), 0.2 M KCl, 0.01 M $MgCl_2$ and 40% (v/v) ethanol; the amounts of ribosomes and substrates are indicated in the figure legends.

The transfer reaction in the presence of cytidine 5'-phosphate was done at 0°C for 10 min. In the absence of cytidine 5'-phosphate the reaction was performed at 0°C for 20 min. Reaction mixture (150 μl) contained: 1 mM pA-Met←f, pA-Phe←f or pA-Leu←f, 7×10^{-7} M ribosomes and 8×10^{-8}–2.4×10^{-7} M C-A-C-C-A-[^{14}C]Phe. The reaction products were extracted with ethyl acetate and the radioactivity was determined [2,3]. Calculation of K_a, K_m, α and k_{cat} were done as in [14,17].

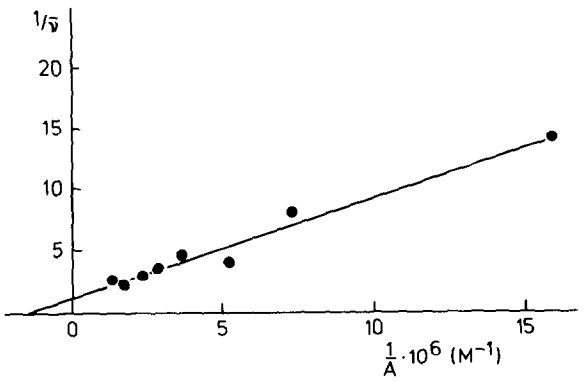

Fig.1. Plot of $1/\bar{\nu}$ vs $1/A$ for the binding of C-A-C-C-A(3'NH-[^{14}C]-Phe←Ac). The amount of substrate was varied from 3.1–35.7 pmol; the amount of ribosomes was 12.5 pmol. $\bar{\nu}$, av. no. substrate molecules bount/ribosome; A, concentration of unbound substrate.

3. Results

3.1. *The binding of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) to ribosomes*

In fig.1 the measurements used for the determination of K_a of the binding of the C-A-C-C-A(3'NH-[^{14}C]Phe Ac) fragment to the ribosomes are presented. The ratio of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac): ribosome was varied from 0.25–2.9. The K_a calculated from this data is 1.4×10^6 M^{-1} and the fraction of ribosomes capable of binding the substrate α is 85%. The statistical treatment of a number of experiments gives $K_a = 1.3 (\pm 0.26) \times 10^6$ M^{-1} and $\alpha = 80 \pm 20\%$.

3.2. *Binding of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) to ribosomes in the presence of acceptor substrates.*

Fig.2 shows the binding of C-A-C-C-A(3'NH-[^{14}C]-

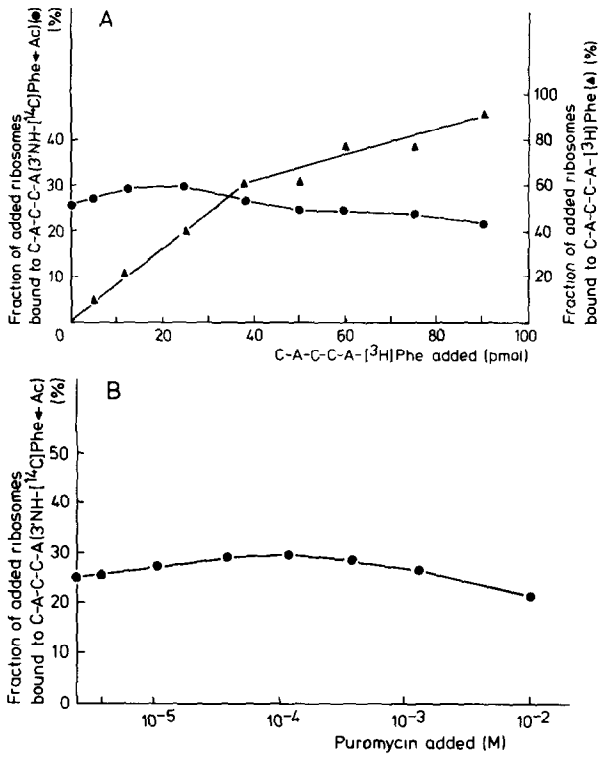

Fig.2. The C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) binding in the presence of increasing amounts of C-A-C-C-A-[^{3}H]Phe (A) and puromycin (B). Incubation mixture contained 12.15 pmol ribosomes and 15 pmol donor substrate.

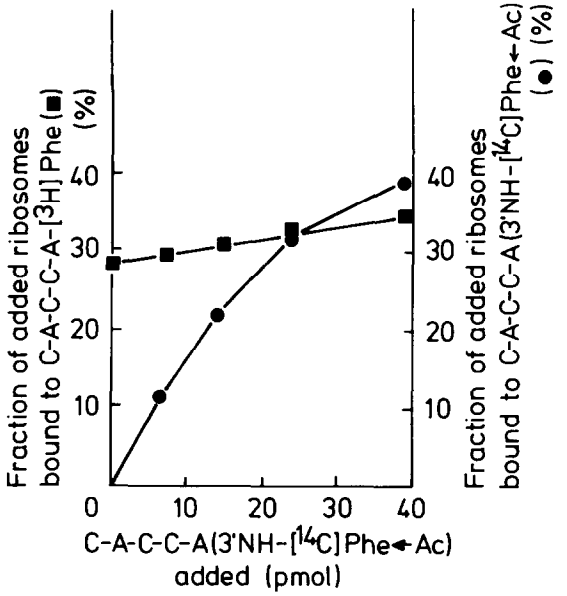

Fig.3. Binding of C-A-C-C-A-[^{3}H]Phe in the presence of increasing amounts of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac). Incubation mixture contained 17 pmol ribosomes and 18.3 pmol acceptor substrate.

Phe←Ac) in the presence of increasing amounts of C-A-C-C-A-[^{3}H]Phe (A) and puromycin (B). It can be seen that no significant effect of acceptor site substrates on C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) binding was observed even when ribosomes were saturated with C-A-C-C-A-[^{3}H]Phe up to 90%. For C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) K_a was 1.50 (± 0.44) X 10^6 M^{-1} and α was 80 ± 20%. These two parameters were practically the same independently of the presence or absence of the acceptor substrate.

3.3. *The binding of C-A-C-C-A-[^{3}H]Phe and C-A-C-C-A-[^{14}C]Val to ribosomes in the presence of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac), pA(3'NH-Met←f) and cytidine 5'-phosphate*

Fig.3 shows the binding of acceptor site substrate, C-A-C-C-A-[^{3}H]Phe, in the presence of increasing amounts of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac). The saturation of ribosomes with C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) was up to 40%. There is no significant stimulation of C-A-C-C-A-[^{3}H]Phe binding in the presence of C-A-C-C-A(3'NH-[^{14}C]Phe←Ac); for C-A-C-C-A-[^{3}H]Phe K_a = (5.0 ± 2.0) X 10^5 M^{-1} and α = 70 ± 10% either in the presence of C-A-C-C-A(3'NH-[^{14}C]-Phe←Ac) or in its absence.

Fig.4 summarizes the data on C-A-C-C-A-[^{3}H]Phe binding in the presence of pA(3'NH-Met←f) or cytidine 5'-phosphate. In both cases the compounds under investigation do not affect the acceptor binding. At concentrations of pA(3'NH-Met←f) or cytidine 5'-phosphate higher than 1 X 10^{-3} M an inhibition of C-A-C-C-A-[^{3}H]Phe binding to the ribosomes can occur. This is due to competition with the acceptor substrate. Similarly the binding of C-A-C-C-A-[^{3}H]Val to ribosomes was stimulated by neither pA(3'NH-Met←f) nor cytidine-5'-phosphate (not shown).

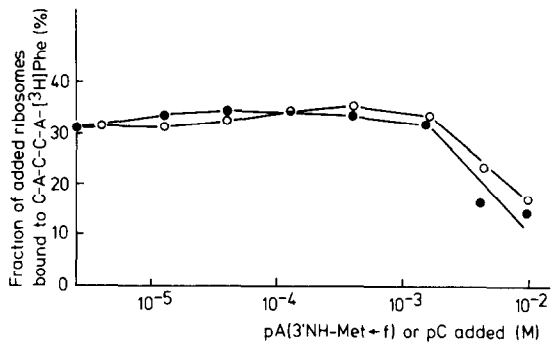

Fig.4. The C-A-C-C-A-[^{3}H]Phe binding in the presence of increasing amounts of pA(3'NH-Met←f) (○) or pC (●). Each tube contained 17 pmol ribosomes and 18.3 pmol C-A-C-C-A-[^{3}H]Phe.

3.4. K_m *and* k_{cat} *of the reactions of model substrates*

K_m and k_{cat} of the ribosomal reaction of the model peptide donors with C-A-C-C-A-[^{14}C]Phe are summarized in table 1. The K_m values are about the same in the presence or absence of cytidine 5'-phosphate. However, the k_{cat} values were stimulated by 1.5 orders of magnitude by the presence of cytidine 5'-phosphate.

4. Discussion

As seen in fig.1 C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) is bound only to a single ribosomal site. It can be confirmed that this site is the donor site. Since the fragment C-A-C-C-A(3'NH-[^{14}C]Phe←Ac) does not inhibit the binding of acceptor substrate C-A-C-C-A-[^{3}H]Phe, its binding is directed to the P-site of the ribosomal peptidyl transferase center. In addition we have shown that the fragments of peptidyl-tRNA C-C-A(3'NH-Leu←f), C-A(3'NH-Met←f) and pA(3'NH-Met←f) are bound to the donor site of the peptidyl transferase centre [14].

Table 1
Effect of cytidine 5′-phosphate (pC) on k_{cat} and K_m in the reaction of model donors with C-A-C-C-A-[^{14}C]Phe

Substrate	With pC		Without pC	
	$K_M(M^{-1} \times 10^7)$	$k_{cat}(s^{-1} \times 10^4)$	$K_m(M^{-1} \times 10^7)$	$k_{cat}(s^{-1} \times 10^6)$
pA-Met←f	10	10	3–10	20–50
pA-Leu←f	2–2.5	2–3	1– 3	5–10
pA-Phe←f	2–2.5	1–2	<1	<5

Fig.2–4 show that under the conditions used the acceptor substrate of the peptidyl transferase centre bound to the acceptor site has practically no effect on K_a and α of the donor substrate and vice versa. At the same time the fact that k_{cat} of the reactions of model donors with C-A-C-C-A-Phe is increased by 1.5 orders of magnitude (table 1) in the presence of cytidine 5′-phosphate implies that this nucleotide leads to the conversion of a low-activity ribosomal complex into high-activity one. Probably the binding of cytidine 5′-phosphate produces some conformational changes in the peptidyl transferase centre facilitating the transpeptidation reaction.

It is possible that the cytidine 5′-phosphate binding locus is not the only area that produces the conformational changes in the peptidyl transferase centre. However, there is no area of binding for the cytidylic residue or residues in the acceptor site capable of transforming a low activity complex into a high activity one. This follows from the fact that cytidine 5′-phosphate does not stimulate the activity of model acceptors (puromycin [1,2] or 3′(2′)-*O*-valyl-adenosine, A-Val; not shown).

These results clearly show that the peptidyl transferase centre is not a rigid matrix binding only its substrates, but it fulfills a more complex function and plays an active role in the process of transpeptidation.

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