THE DONOR SITE OF THE PEPTIDYLTRANSFERASE CENTER OF RIBOSOMES

Equilibrium association constants of model substrates and inhibitors

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1. Introduction

There are several publications concerning determination of the binding constants (K_{ass}) of model acceptor substrates of the peptidyltransferase center of *Escherichia coli* ribosomes such as CACCA-Phe [1,2] and puromycin [3] However, no quantitative data on binding of model substrates to the donor site have been reported so far

Here we report determination of the equilibrium binding constants of the pentanucleotide fragment of peptidyl-tRNA CACCA-Leu←Ac and several other model peptide donors [4,5] and donor site inhibitors of the peptidyltransferase center of *E coli* ribosomes It has been found that K_{ass} changes insignificantly from the pentanucleotide to the trinucleotide model substrates and inhibitors of the donor site and decreases 2--3 orders of magnitude, upon transition to their di- and mononucleotide analogs

2. Materials and methods

2 1. E coli *MRE-600 ribosomes*

These were isolated by two methods (A) by treatment with puromycin as in [6], (B) by centrifugation in a sucrose gradient in a zonal rotor, according to [7] The ribosomes isolated by method A contained ~20% of 50 S subunits Method B allowed the production of nearly pure 70 S ribosomes (established by sedimentation in the sucrose density gradient)

2 2 *CACCA-[^{14}C]Leu←Ac*

This was obtained from [^{14}C] Leu-tRNA (*E coli*) by acetylation with Ac_2O and subsequent hydrolysis with T_1-ribonuclease Specific radioactivity of [^{14}C]leucine was 348 mCi/mmol (Amersham, England) Purification of CACCA-[^{14}C]Leu←Ac was done by electrophoresis on 3 MM Whatman paper, 3500 V, 100 mA, for 2 h TLC on silica gel plates was used to prove that the preparation was free of shorter labeled fragments, e g , CCA-[^{14}C] Leu←Ac, *n*-BuOH H_2O (85 15, v/v) was used in the first direction, *tert*-BuOH 0 075 M formic acid (pH 4 8) adjusted by NH_4OH (1 1, v/v) in the second Completeness of acetylation and safe keeping of the preparation were tested by paper chromatography in the system *n*-BuOH–AcOH–H_2O (5 2 3, v/v/v), CACCA-[^{14}C]-Leu←Ac R_f = 0, Ac-[^{14}C] Leu R_f = 0 9, [^{14}C] Leu R_f = 0 7

Substances pA-Met←f, pA-Met←Ms and CpA-Met←f were obtained as in [8], pA(3'NHMet←Ms), CpA(3'NHMet←f) and CpCpA(3'NHLeu←Ms) according to [9]

2 3 *The K_{ass} of CACCA-[^{14}C]Leu←Ac to ribosomes*

Adsorption isotherms of CACCA-[^{14}C] Leu←Ac on the 70 S ribosomes were studied in the conditions of fragment reaction [10] The 0 1 ml incubation mixture

Abbreviations CACCA-Leu←Ac and CACCA-Phe, pentanucleotide 3'-fragments of *N*-acetylleucyl-tRNA and phenylalanyl-tRNA, respectively, pA-Met←f and pA(3'NHMet←Ms), 5'-phosphates of 2'(3')-*O*-(*N*-formylmethionyl)adenosine and 3'-*N*-(*N'*-methanesulfonylmethionyl)amino-3'-deoxyadenosine, respectively, CpA-Met←f and CpA(3'NHMet←f), cytidilyl-(3'→5')-2'(3')-*O*-(*N*-formylmethionyl)adenosine and cytidilyl-(3'→5')-3'-*N*-(*N'*-formylmethionyl)amino-3'-deoxyadenosine, respectively, CCA(3'NHLeu←Ms), cytidilyl-(3'→5')cytidilyl-(3'→5')-3'-*N*-(*N'*-methanesulfonylleucyl)amino-3'-deoxyadenosine

contained 0.06 M Tris–HCl buffer (pH 7.5), 0.2 M KCl and 0.01 M $MgCl_2$, 50% ethanol, the amounts of ribosomes and fragments are indicated in the figure legends. The reaction was run in weighed, conical test-tubes (2 × 0.2 cm). The samples were protected with parafilm to prevent evaporation, and incubation was run at 0°C for 60 min. The suspension was then centrifuged for 10 min at 5000 rev./min in a K-23 centrifuge (Carl Janetzki, DDR) at 0°C. In these conditions a complete precipitation of ribosomes was achieved due to the aggregation of ribosomes in 50% ethanol. After centrifugation the supernatant contained ⩽3–4% of the total A_{260} material. The amount of fragment bound to ribosomes was determined as a difference of the total amount of CACCA-[^{14}C]-Leu←Ac in the sample and the amount of free fragment. The latter was found by measuring the radioactivity in the weighed aliquot of supernatant (50% of the total supernatant) counted in 5 ml of dioxane scintillator. The total amount of fragment was found by summation of the measured radioactivity and the radioactivity in the residual supernatant with the precipitate. The amount of fragment bound is the difference between the total fragment in the sample and the amount of free fragment. The binding constants were found using a double reciprocal plot for the binding of CACCA-[^{14}C]Leu←Ac.

2.4. *The* K_{ass} *of substrate analogs and inhibitors*

The same approach was used to determine the app. K_{ass} in the presence of the analogs. The binding constants of these compounds (K_B) were calculated as:

$$K_B = \frac{1}{B} \cdot \frac{K_A - K_{A'}}{K_{A'}}$$

where K_A and K_B are the association constants of compounds A (fragment CACCA-Leu←Ac) and B (inhibitor); $K_{A'}$ the apparent association constant of A at the given concentration of B.

3. Results

3.1. *Binding of CACCA-[*^{14}*C]Leu←Ac to 70 S ribosomes*

Two series of experiments were done to determine

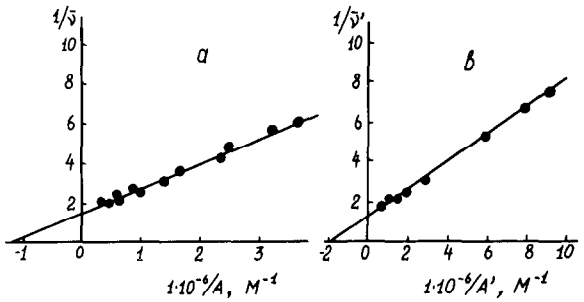

Fig.1. Binding of CACCA-[^{14}C]Leu←Ac to ribosomes double-reciprocal plot (experimental conditions are given in the text). (a) Concentration of ribosomes 3.6 × 10^{-7} M; concentration of fragment was varied from 1.2 × 10^{-7} to 1 × 10^{-6} M. $\bar{\nu}$, the average number of molecules of fragment bound per ribosome. A, concentration of free fragment. (b) Concentration of fragment was 1 × 10^{-7} M, concentration of ribosomes was varied from 3.8 × 10^{-7} to 1.2 × 10^{-6} M. $\bar{\nu}'$, the total part of CACCA-[^{14}C]Leu←Ac capable of associating with ribosomes; A', concentration of free ribosomes.

K_{ass}. In the first series a constant amount of ribosomes was used, and the concentration of fragment varied. In the second, a constant amount of fragment was titrated with ribosomes. Figure 1a,b summarizes the results of the determination of K_{ass} of CACCA-[^{14}C]Leu←Ac to the ribosomes isolated by method B. The results are presented as a double reciprocal plot. As is seen in fig.1a, K_{ass} = 1.2 × 10^6 M^{-1}, and 0.6–0.7 of the fragment molecules are bound to a ribosome, i.e., 60–70% of the ribosome preparation binds the fragment actively. Similar determination of K_{ass} for ribosomes isolated by method A gave an identical K_{ass}, but the amount of active ribosomes was 25–40% (data not shown).

When the amount of CACCA-Leu←Ac was constant and the concentration of ribosomes varied (fig.1b), we found K_{ass} ≈2 × 10^6 M^{-1}. It should be noted that in our experiments we recovered only 80–90% of the active fragment, and 10–20% did not bind to ribosomes. This may be explained by a partial hydrolysis of the fragment during the experiment.

The described determinations of K_{ass} were done at a fragment concentration of 5 × 10^{-8} to 5 × 10^{-7} M. However, if the fragment concentration was increased to 5 × 10^{-6} M, another slope with K_{ass} ≈7 × 10^4 M^{-1} appeared on the curve; up to two molecules of fragment were bound per ribosome.

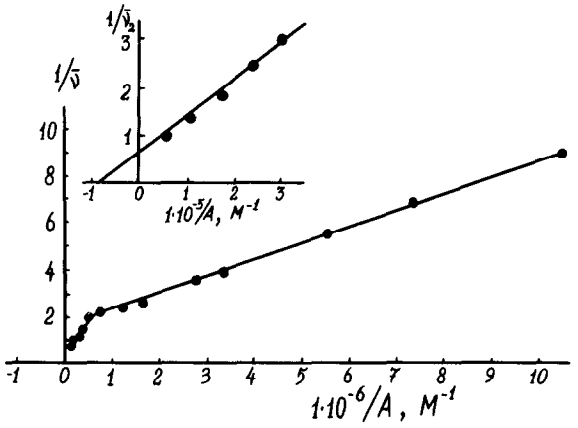

Fig 2 Binding of CACCA-[^{14}C]Leu←Ac to ribosomes Concentration of ribosomes 3 6 × 10^{-7} M, concentration of fragment was varied from 1 2 × 10^{-7} to 7 × 10^{-6} M Inset shows the binding of CACCA-[^{14}C]Leu←Ac to the second site of the ribosome The data were calculated from the results presented on the lower curve and from the equation $\bar{\nu} = \bar{\nu}_1 + \bar{\nu}_2$ where $\bar{\nu}_1$ and $\bar{\nu}_2$ are the average number of CACCA-[^{14}C]Leu←Ac molecules bound per donor site and the second (probably the acceptor) site, respectively

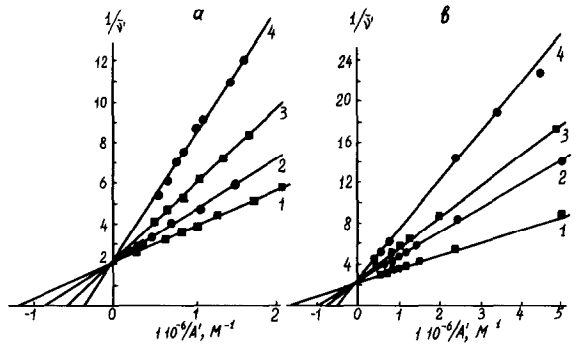

Fig.3 Plot of $1/\bar{\nu}'$ versus $1/A$ for the binding of CACCA-[^{14}C]Leu←Ac to ribosomes in the presence of inhibitors pA-Met←f (a) and CpA-Met←f (b) (a) Control in the absence of inhibitor (1), in the presence of pA-Met←f 1 × 10^{-3} M (2), 2 5 × 10^{-3} M (3) and 5 × 10^{-3} (4), CACCA-[^{14}C]Leu←Ac 1.2 × 10^{-7} M, the concentration of ribosomes was varied from 3 8 × 10^{-7} to 1 9 × 10^{-6} M (b) Control in the absence of inhibitor (1), in the presence of CpA-Met←f 1 1 × 10^{-4} M (2), 3.8 × 10^{-4} M (3), 1 × 10^{-3} M (4) CACCA-[^{14}C]Leu←Ac 1 2 × 10^{-7} M, the concentration of ribosomes was varied from 2 2 × 10^{-7} to 1 8 × 10^{-6} M

3 2 *The* K_{ass} *for pA-Met←f and CpA-Met←f*

K_{ass} of CpA-Met←f and pA-Met←f were found from their competition with CACCA-[^{14}C]Leu←Ac

The binding of CACCA-[^{14}C]Leu←Ac to ribosomes at several concentrations of inhibitors CpA-Met←f and pA-Met←f are given as double reciprocal plots in fig.3a,b. For pA-Met←f K_{ass} was (4 3 ± 0.5) × 10^2 M^{-1}, for CpA-Met←f (2 ± 0-56) × 10^3 M^{-1}. Figure 3 shows that pA-Met←f and CpA-Met←f are competitive inhibitors of CACCA-Leu←Ac

3.3. *The* K_{ass} *for pA(3'NHMet←Ms) and CpA(3'NHMet←f)*

Inhibition of the reaction of CACCA-[^{14}C]Leu←Ac and puromycin [11] by pA-Met←f and pA(3'NHMet←Ms) is shown in fig 4 Both the substances inhibit the process in almost similar fashion Determination of K_{ass} made as described in section 3 2 (fig.5a,b) demonstrated K_{ass} to be very close to K_{ass} of pA-Met←f and CpA-Met←f, table 1. When larger concentations of pA(3'NHMet←Ms) were used, the intersection sometimes departed from the ordinate which was, presumably, due to experimental errors rather than to another type of inhibition

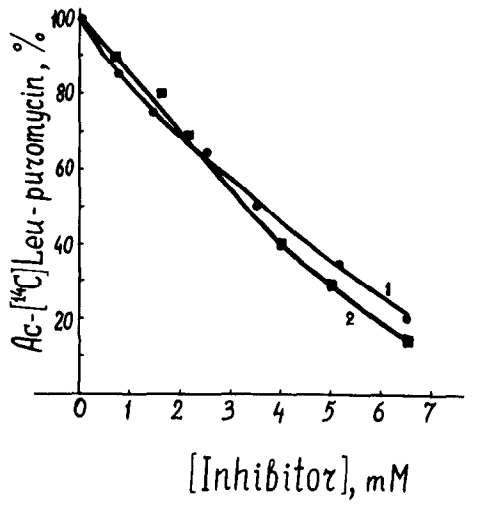

Fig 4 Inhibition of CACCA-[^{14}C]Leu←Ac–puromycin reaction by pA-Met←f (1) and pA(3'NHMet←Ms) (2) Incubation mixture contained 3 3 mM puromycin, 3 A_{260} units of ribosomes and 1 1 × 10^{-7} M CACCA-[^{14}C]Leu←Ac, total vol 0 15 ml Incubation was run for 1 h, 0°C 70% of CACCA-[^{14}C]Leu←Ac reacted with puromycin without the inhibitor

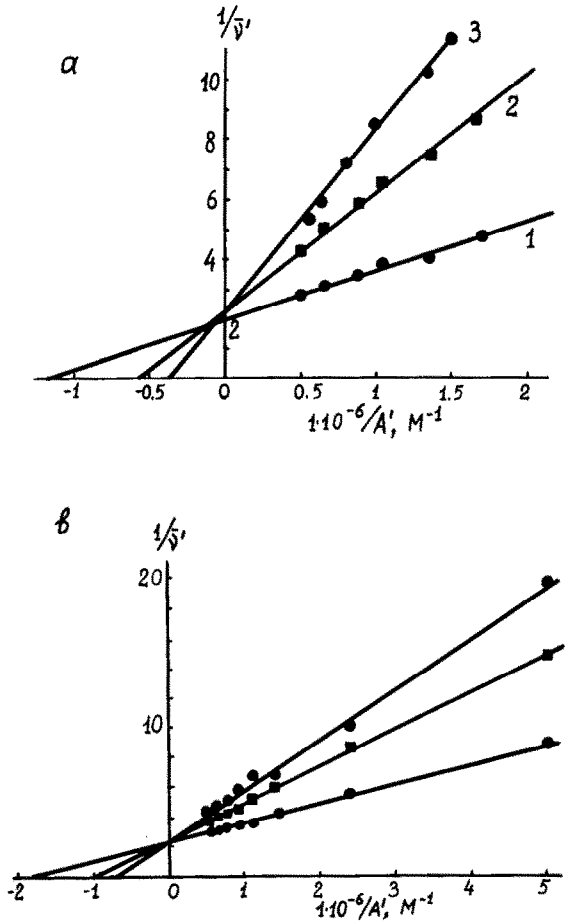

Fig.5. Competitive inhibition of CACCA-[^{14}C]Leu←Ac binding with pA-Met←f and pA(3'NHMet←Ms) (a), CpA-Met←f and CpA(3'NHMet←f) (b). (a) Binding of CACCA-[^{14}C]-Leu←Ac in the absence of inhibitors (1), in the presence of 2.5×10^{-3} M pA(3'NHMet←Ms) (2) and 5×10^{-3} M pA-Met←f (3). Concentration of CACCA-[^{14}C]Leu←Ac 1.2×10^{-7} M, concentration of ribosomes was varied from 3.8×10^{-7} to 1.9×10^{-6} M. (b) Binding of CACCA-[^{14}C]-Leu←Ac in the absence of inhibitors (1), in the presence of 5×10^{-4} M CpA-Met←f (2) and of 2.6×10^{-4} M CpA-(3'NHMet←f). Concentration of CACCA-[^{14}C]Leu←Ac 1.2×10^{-7} M, concentration of ribosomes was varied from 2.2×10^{-7} to 1.8×10^{-6} M.

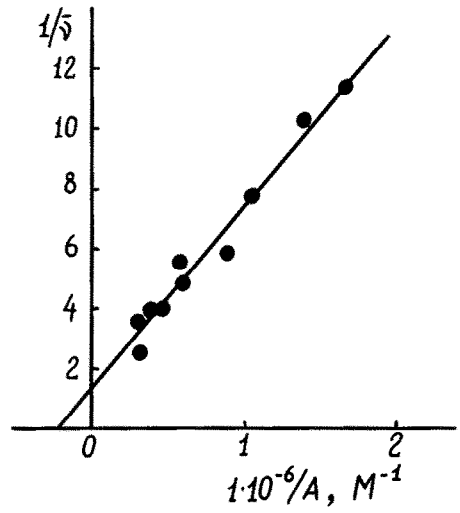

Fig.6. The plot of $1/\bar{v}$ versus $1/A$ for CCA(3'NH-[^{14}C]-Leu←Ms) binding. Concentration of CACCA-[^{14}C]Leu←Ac was varied from 1×10^{-7} to 2×10^{-6} M.

Table 1
Association constants of model substrates and inhibitors of the donor site

Donor site			Acceptor site	
Compound	K_{ass} · M	Compound	K_{ass} · M (20% alcohol)	Ref.
CACCA-Leu←Ac	[a](1.7 ± 0.1) × 10^6	CACCA-Phe	2.76×10^7	[1]
	b(7.0 ± 0.7) × 10^4		9.90×10^7	[2]
CCA(3'NHLeu←Ms)	(2.6 ± 0.8) × 10^5			
CpA-Met←f	(2.0 ± 0.56) × 10^3			
CpA(3'NHMet←f)	(5.4 ± 1.2) × 10^3			
pA-Met←f	(4.3 ± 0.5) × 10^2			
pA(3'NHMet←Ms)	(3.6 ± 0.5) × 10^2	Puromycin	2.4×10^3	[3]

[a] K_{ass} for the donor site
[b] K_{ass} for the other site of the peptidyltransferase center (probably, for the acceptor site

3.4. *The binding constant for CCA(3'NH-[^{14}C]Leu←Ms)*

Figure 6 shows the determination of K_{ass} of CCA(3'NH-[^{14}C]Leu←Ms) obtained as for that of CACCA-[^{14}C]Leu←Ac, it was found to be $(2\,6 \pm 0\,8) \times 10^5$ M^{-1}

4 Discussion

The ethanol precipitation method used in our work to find the equilibrium binding constant of CACCA-Leu←Ac was described in [12] where it was used to determine antibiotic binding constants The values obtained by that method and by equilibrium dialysis appeared to be similar Applicability of the method to the problem under consideration was proved by the following experiments

(1) Centrifuging of the CACCA-Leu←Ac fragment in the absence of ribosomes showed that the fragment itself does not precipitate in the chosen conditions
(2) Centrifuging of CACCA-Leu←Ac in the presence of an excess (1×10^{-2} M) of the competing pA-Met←f and ribosomes proved that the fragment was not co-precipitated with ribosomes
(3) In the range of the concentrations used the yield of the reaction product was directly proportional to the ribosome concentration

The presence of ethanol increased the binding of model substrates to the donor site of the peptidyl-transferase center of ribosomes [13,14] Determination of K_{ass} of CACCA-Leu←Ac and of the compounds mentioned here was also made in 50% methanol and the results were similar to those obtained in 50% ethanol (A V Kosenjuk, data not shown). It should be noted that K_{ass} of CACCA-Leu←Ac was almost identical for ribosomes isolated by different methods, whereas the amount of active ribosomes isolated by the gradient sedimentation method was twice as much

Measurement of CACCA-Leu←Ac binding revealed the presence of two binding sites with K_{ass} = $1\,7 \times 10^6$ M^{-1} and $K_{ass} \approx 7 \times 10^4$ M^{-1} The first constant was obviously associated with the donor site because at the concentrations of fragment used ($<5 \times 10^{-7}$ M) 70–80% of the acetylleucine were transfered from CACCA-Leu←Ac onto puromycin The other site was, presumably, the acceptor one because at appreciable concentrations of CACCA-Leu←Ac one could observe effective inhibition of transfer of the AcLeu-residue from CACCA-Leu←Ac onto puromycin (see [15])

When determining the K_{ass} of CACCA-Leu←Ac and that of CpA-Met←f and pA-Met←f, the quality of these compounds ought to be under very strict control as they are capable of undergoing hydrolysis and alcoholysis These difficulties, however, are not encountered in the work with 3'-deoxy-3'-amino analogs of substrates CCA(3'NHLeu←Ms), CpA(3'-NHMet←f) and pA(3'NHMet←Ms) which are resistant to the conditions of reaction Figure 4 gives the results of comparative inhibition of the CACCA-[^{14}C]Leu←Ac - puromycin reaction by pA-Met←f and pA(3'NHMet←Ms), CpA(3'NHMet←f) is similar in its behavior to CpA-Met←f

Using the methane sulfonyl group instead of the formyl group in the model substrates and inhibitors of the donor site does not affect their donor activity or their K_{ass} values The methane sulfonyl groups, however, are chemically more stable and racemization of amino acid is excluded during synthesis of model substrates and inhibitors

The results obtained show that 3'-deoxy-3'-amino compounds are bound to the donor site and compete with the pentanucleotide fragment The data on K_{ass} support this view Thus, the 3'-deoxy-3'-amino analogs of model substrates appear to be new model inhibitors of the donor site whose K_{ass} is practically identical to the K_{ass} of model substrates of the donor site

Analyzing the column of K_{ass} of peptide model donors and peptide inhibitors in table1, one should take note of the sharp increase of K_{ass} observed at the transition from the dinucleosidephosphate to the trinucleosidediphosphate derivative (K_{ass} is changed by 2 orders of magnitude) This explains the results obtained earlier [16,17] on high peptide donor activity of the compounds having three or more nucleotide residues in the oligonucleotide moiety of the substrate, which may be caused either by a firmer attachment due to the presence of extra interaction sites, or by the occurrence of a more favourable conformation as a whole

Table 1 presents the K_{ass} of substrates and inhibitors of the donor site determined by us and the K_{ass} of the substrates of the acceptor site described in

literature. It may be seen that a certain parallelism in the change of K_{ass} of substrates upon transition from pentanucleotide substrates to their mononucleotide analogs is characteristic of both the columns in table 1. A final conclusion, however, can only be made after systematic analysis of K_{ass} of the acceptor site model substrates as has already been done for the donor site.

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