KINETIC CONSTANTS FOR MODEL SUBSTRATES OF PEPTIDYLTRANSFERASE DONOR SITE OF *ESCHERICHIA COLI* RIBOSOMES

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1. Introduction

The substrate specificity of the peptidyltransferase center of *E. coli* ribosomes has been investigated in several laboratories. These studies enable a general description to be made of the interaction of substrates with the donor and acceptor sites of ribosomes [1,2].

To further advance understanding of the mechanism of functioning of the peptidyltransferase center as an enzymatic system, enzyme kinetic methods are required to make quantitative comparisons of interactions of different model substrates with their appropriate binding sites. Kinetic constants for acceptor site substrates have been reported [3–5] but at the time of writing no such data have been published for the donor site. For determination of K_m and K_i of model substrates we used the reaction suggested in [6]. This reaction of the 3′-terminal pentanucleotide fragment of peptidyl-tRNA CACCA-Leu←Ac with puromycin occurs on ribosomes in the absence of a template.

Abbreviations: CACCA-Leu←Ac and ACCA-Tyr, the 3′-terminal fragments of AcLeu-tRNA and Tyr-tRNA, respectively; A-Phe, 2′(3′)-*O*-phenylalanyladenosine; pA-Met←f, pA-Leu←f and pA-Phe←f, 2′(3′)-*O*-(*N*-formyl)aminoacyl derivatives of adenosine 5′-phosphate; CpA-Met←f and CpA-Met←Ms, cytidilyl-(3′→5′)-2′(3′)-*O*-(*N*-formylmethionyl)-adenosine and cytidilyl-(3′→5′)-2′(3′)-*O*-(*N*-methanesulfonyl-methionyl)adenosine, respectively; pA(3′NHMet←Ms), 3′-(*N*-methanesulfonylmethionyl)amino-3′-deoxyadenosine 5′-phosphate; CpA(3′NHMet←f) and CpA(3′NHPhe), cytidilyl-(3′→5′)-3′-(*N*-formyl)amino-3′-deoxyadenosine and cytidilyl-(3′→5′)-3′-(*N*-phenylalanyl) amino-3′-deoxy-adenosine, respectively; A(3′NHPhe), 3′-(*N*-phenylalanyl)-amino-3′-deoxyadenosine

We have determined K_m (or K_i) for a number of donor site model substrates and have shown that K_m (or K_i) is decreased by 3 orders of magnitude upon transition from the dinucleotide to the pentanucleotide derivative. Besides, we found that the analogs of model substrates with an acylamino acid attached to the nucleotide residue via an amide (not an ester) bond are competitive inhibitors for CACCA-Leu←Ac in the reaction with puromycin and their affinities are close to those of the substrates of corresponding structures. The K_m of CACCA-Leu←Ac is 1–1.5 orders of magnitude smaller than the K_s of the same compound [7]. This fact may be explained by the positive influence of substrate binding to the acceptor site on complex formation of CACCA-Leu←Ac with the donor site.

2. Materials and methods

Isolation of *E. coli* MRE-600 ribosomes, their activation prior to the reaction and obtaining of CACCA-[^{14}C]Leu←Ac were as described in [7]. The 50 S subunits of *E. coli* MRE-600 were a kind gift of Dr S. V. Kirillov. The 50 S subunits were activated by heating at 40°C for 10 min. The inhibitors were synthesized by A. V. Azhayev and S. V. Popovkina [8]. Puromycin dihydrochloride (Calbiochem), [^{14}C]-leucine of specific radioactivity 348 mCi/mmol (Amersham, England) and 190 mCi/mmol (ÚVVVR, Czechoslovakia) were used.

The incubation mixture contained: 20 mM $MgCl_2$, 40 mM Tris–HCl ($pH_{20°C}$ 7.4), 200 mM KCl and 50% methanol, if no other indications are given. The concentrations of ribosomes, puromycin, CACCA-[^{14}C]-

Leu←Ac and inhibitors are given in the legends to the figures. The reaction was run at 0°C and terminated by adding KOH to a final concentration of 0.7 M. The side product Ac-[^{14}C]LeuOMe was hydrolyzed in 0.7 M KOH at 40°C for 40 min, and then Ac-[^{14}C]-Leu-puromycin was extracted with ethylacetate. The extract was washed with water, dried with Na_2SO_4, and the radioactivity measured in a toluene + methyl cellosolve scintillation solution on a counter SL-30 (Intertechnique, France). Efficiency of counting was 90%.

3. Results

3.1. *Determination of* K_m *for CACCA-[*^{14}*C]Leu←Ac at different methanol concentrations*

To choose the range of methanol concentration for K_m determination, the yield of the reaction at different methanol concentrations was measured (fig.1). Fig.2 shows a typical evaluation of K_m for CACCA-[^{14}C]Leu←Ac under similar conditions. Estimation was made within 7 min when the product yield was linear with time. The reaction mixture without puromycin was used as a blank. As is shown in fig.1 and 2 and table 1, the optimal concentration of methanol was 45–50%, which somewhat deviates from Monro and Marcker's results [6]. It is obvious from fig.2 that methanol stimulates the transferase reaction due to the increase of affinity of the donor site substrates for the peptidyltransferase center.

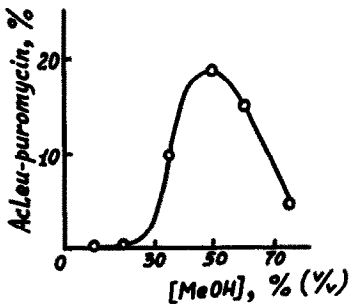

Fig.1. Dependence of the CACCA-[^{14}C]Leu←Ac–puromycin reaction yield on methanol concentration. Concentration of ribosomes 1.06 × 10^{-7} M, puromycin 1.03 mM, CACCA-[^{14}C]Leu←Ac 3.7 × 10^{-8} M. Incubation 16 min. The amount of CACCA-[^{14}C]Leu←Ac introduced into reaction was taken as 100%.

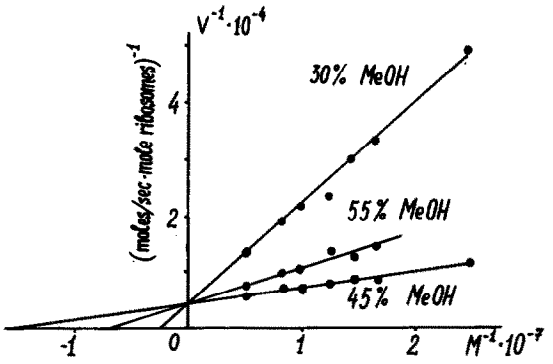

Fig.2. Effect of methanol concentration on rate of the reaction of CACCA-[^{14}C]Leu←Ac with puromycin.

At CACCA-Leu←Ac concentrations higher than 5 × 10^{-7} M we observed a linear increase of reciprocal velocity with increasing substrate concentration. This proves that an inactive complex of ribosomes with two molecules of CACCA-Leu←Ac is formed. One molecule being bound to the donor site, and the other, presumably, to the acceptor site.

Table 1

K_m and V_{max} of the reaction of CACCA-Leu←Ac with puromycin at different methanol concentrations

Methanol concentration % (v/v)	K_m (M × 10^8)	V_{max} × 10^4 mol/(sec. mol ribosomes)
30	42	2.4
33	26.7	3.6
45	6.7	2.4
50	7.4	2.9
55	14	2.4

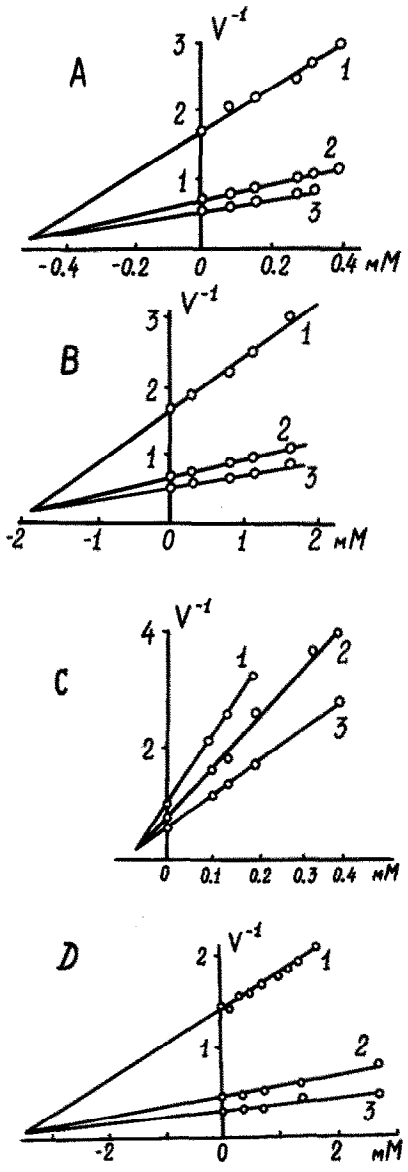

Fig.3. Inhibition of the reaction of CACCA-[^{14}C]Leu←Ac with puromycin by: (A) pA(3'NHMet←Ms) and (B) pA-Phe←f; concentration of ribosomes 1.96 × 10^{-7} M, puromycin 1.02 mM, CACCA-[^{14}C]Leu←Ac 3.75 × 10^{-8} M (1); 1.13 × 10^{-7} M (2); 1.50 × 10^{-7} M (3); (C) CpA(3'NHMet←f), concentration of ribosomes 1.16 × 10^{-7} M, puromycin 1.03 mM, CACCA-[^{14}C]Leu←Ac 3.8 × 10^{-8} M (1); 5.7 × 10^{-8} M (2); 1.14 × 10^{-7} M (3); (D) pA-Met←f, concentration of ribosomes 1.96 × 10^{-7} M, puromycin 1.02 mM, CACCA-[^{14}C]Leu←Ac 3.0 × 10^{-8} M (1); 2.5 × 10^{-7} M (2); 3.0 × 10^{-7} M (3). Reaction velocity (v) in mol × 10^{-4} of AcLeu-puromycin synthesized in 1 s/1 mol of ribosomes. Concentration of the inhinitor is indicated on the abscissa.

One can see that the experimental curves obtained at different concentrations of methanol intersect on the ordinate, showing that both V_{max} and the amount of active ribosomes are independent of the methanol concentration.

3.2. *Measurements of* K_i *for other model substrates and inhibitors*

Fig.3 and table 2 represent the results of studies on K_i of the model substrates pA-Met←f, pA-Phe←f, CpA-Met←f and CpA-Met←Ms and the inhibitors pA(3'NHMet←Ms), CpA(3'NHMet←f) and pA(3'NHGly ←Ms) in the reaction of CACCA-[^{14}C]Leu←Ac with puromycin. Table 2 shows that the inhibition con-

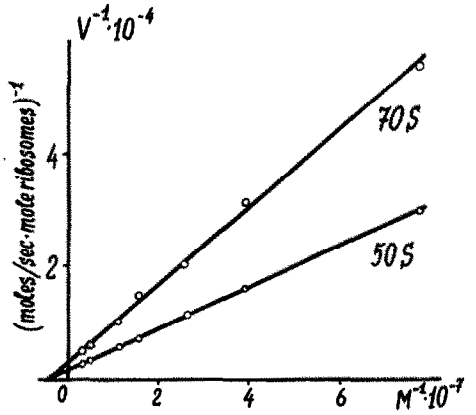

Fig.4. Double reciprocal plot of the dependence of the reaction rate of Ac-Leu-puromycin formation on CACCA-[^{14}C]-Leu←Ac concentration for reactions catalyzed by 70 S ribosomes and 50 S subunits. Concentration of ribosomes 3.34 × 10^{-7} M, 50 S subunits 3.07 × 10^{-7} M, puromycin 0.94 mM, 33% methanol.

Table 2
Inhibition constants of model substrates and inhibitors in the reaction of CACCA-Leu←Ac with puromycin

Inhibitor	K_i (M × 10^3)
CpA-Met←f	0.16
CpA-Met←Ms	0.11
CpA(3'NHMet←f)	0.066
pA-Met←f	3.60
pA(3'NHMet←Ms)	0.52
pA(3'NHGly←Ms)	1.40
pA-Leu←f	1.50
pA-Phe←f	1.95

stant depends on the length of the nucleotide component: the affinity of dinucleoside phosphate esters and amides for the donor site is one order of magnitude higher than that of the corresponding adenosine 5′-phosphate derivatives. A larger degree of inhibition is demonstrated for the 3′-amide derivatives as compared to those of 2′(3′)-ester.

3.3. *Comparison of the reactions catalyzed by the 70 S ribosomes and 50 S subunits*

Fig.4 presents the results for the reaction of CACCA-[^{14}C]Leu←Ac with puromycin catalyzed by 70 S ribosomes and by 50 S subunits. One can also see that K_m is practically the same in both cases.

4. Discussion

The results obtained allow one to conclude that the kinetic constants of the model substrates and inhibitors largely depend on the nature of the nucleotide component and to a lesser degree on the character of the amino acid component, the structure of the acyl group and the type of bond between the acyl-amino acid residue and the nucleotide moiety.

First of all, one has to mention the change of K_m (K_i) in the series CACCA-Leu←Ac < CpA-Met←f < pA-Met←f. This correlates with the change of K_{diss} for the same sequence of compounds.

Alteration of some structural elements in the substrates or inhibitors affects K_i of donor substrates and K_i of acceptor substrates but to a different degree (table 3). For example, when the derivatives of dinucleoside phosphate are taken instead of adenylic acid derivatives, K_i is decreased one order of magnitude for the donor site, whereas for the acceptor site somewhat similar structural differences results in K_i being altered by less than a factor of two. The ratio of K_m of CACCA-Leu←Ac to K_i of pA-Met←f is 4×10^3 for the donor substrates whereas the ratio of K_m of ACCA-Tyr to K_i of A-Phe is only 40 for the acceptor substrate. It has to be noted that activities of the amino acid derivatives of adenosine 5′-phosphate are rather close [9].

Estimation of K_{diss} and K_m (K_i) for the model donors and inhibitors was made when the peptidyltransferase center was in different functional states. K_{diss} of the donor substrates was found when the acceptor site was vacant, whereas K_i was estimated when the acceptor site was occupied with puromycin. The ability of the peptidyltransferase center to bind its substrates is preconditioned by the influence of the acceptor and donor sites on one another. This explains why the K_m value is 1–1.5 orders of magnitude less than K_{diss}, which is indirect evidence of a positive action of the peptidyltransferase acceptor site on the donor site.

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Table 3
Comparison of K_m (K_i) for the mono- and the oligonucleotide derivatives (model substrates and inhibitors) of the donor site with those of the acceptor site

Donor site			Acceptor site			
Substance	K_m (K_i) × M	Ratio[a]	Substance	K_m (K_i) × M	Ratio[b]	Ref.
CACCA-Leu←Ac	7.4×10^{-8}		ACCA-Tyr	8×10^{-6}		[3]
pA-Met←f	3.6×10^{-3}	1	A(3′NHPhe)	2.8×10^{-3}	1	[5]
CpA-Met←f	1.8×10^{-4}	0.05	CpA(3′NHPhe)	1.7×10^{-3}	0.61	[5]
pA(3′NHMet←Ms)	5.2×10^{-4}	0.14	A-Phe		1.18	[4]
CpA(3′NHMet←Ms)	6.6×10^{-5}	0.018				

[a] Ratio of K_i of model donors and inhibitors to K_i of pA-Met←f
[b] Ratio of K_i CpA(3′NHPhe) and A-Phe to K_i of A(3′NHPhe)

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