THE ROLE OF THE CYTIDINE RESIDUES OF THE tRNA 3′-TERMINUS AT THE PEPTIDYLTRANSFERASE A- AND P-SITES

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1. Introduction

The 3′-terminal nucleotides of tRNA play a major role in the interaction of both aminoacyl and peptidyl-tRNAs with the acceptor and donor sites of peptidyltransferase [1]. Although the minimal requirement for donor activity is the presence of 2′(3′)-*O*-(*N*-acyl)aminoacyl)adenosine 5′-phosphate [2] at the donor site, and 2′(3′)-*O*-aminoacyladenosine is a minimal acceptor substrate [3], the activity of these simple models in the peptidyltransferase reaction is considerably increased by adding either a cytidine or a cytidine 3′-phosphate residue, respectively. Thus, C-A(fMet) is ~30-times more active as a donor substrate than pA(fMet) [4], and C-C-A(fMet) is still more active [5]. At the acceptor site, C-A-Gly is a good acceptor substrate, whereas A-Gly has no activity [6], and C-A-Phe is ~500-times more active than A-Phe [7].

Cytidine 5′-phosphate strongly stimulates the donor activity of, e.g., pA(fMet), most probably by occupying that part of the donor site of peptidyltransferase which would otherwise bind the penultimate cytidine residue of the 3′-terminus of peptidyl-tRNA, thus simulating the presence of, e.g., pC-A(fMet) [8]. In contrast, the transfer of AcPhe residue from AcPhe-tRNA to puromycin is not significantly influenced by Cp, pC and C-C [9].

Despite this evidence several reports have claimed that the cytidylic acid residues of the acceptor substrate have virtually no role in binding to the A-site of peptidyltransferase [9–11]. We have attempted to resolve these conflicting claims by investigating the effect of several cytidine nucleotides on the donor and acceptor activities of the substrates C-A(AcMet) and C-A-Lys. We conclude that cytidine nucleotides significantly influence the activities of both donor and acceptor substrates. We have also compared the acceptor activities of 2′(3′)-*O*-aminoacyl-derivatives of adenosine and C-A, and found a dramatic increase in the activities of the C-A derivatives as compared to the adenosine derivatives in 7 different cases. It is clear that binding of the cytidine residues of 3′-terminus of tRNA to either the acceptor or the donor site plays a major role in the peptidyltransferase reaction, contrary to the repeated claims [9–11] that there is a 'lack of the effective binding of cytidylic acid to the A site'.

Abbreviations: aa-tRNA, aminoacyl transfer ribonucleic acid; C-A(AcMet), cytidylyl-(3′–5′) 2′(3′)-*O*-(*N*-acetyl-L-methionyl)adenosine, (and similar abbreviations for other dinucleotide derivatives)

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2. Materials and methods

Three times washed ribosomes from *Escherichia coli* MRE-600 cells were prepared as in [7]. *N*-Ac-[^{14}C]Phe-tRNA (spec. act. 0.84 nmol [^{14}C]Phe/mg tRNA) and [^{14}C]Phe-tRNA (spec. act. 0.3 nmol [^{14}C]Phe/mg tRNA) were prepared as in [7]. The chemical synthesis of C-A-Lys and other 2′(3′)-*O*-aminoacyl-dinucleotides has been described [12,13], and that of C-A(AcMet) will appear [4]. pC and C-C were commercial preparations (Sigma, St Louis, MO), cytidine 3′-phosphate was prepared according to [14], and pCp was a kind gift from Dr P. Bhuta, Michigan Cancer Foundation. Assay conditions for the peptidyltransferase reactions are described in the figure legends.

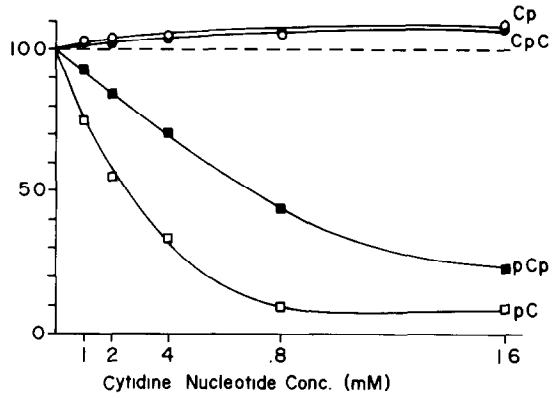

Fig.1. Effect of various cytidine nucleotides on the peptidyltransferase donor reaction using C-A(AcMet) as donor (fragment reaction). Assays were performed as in [2]; a typical reaction mixture contained in 0.05 ml: 0.05 M Tris–HCl (pH 7.4), 0.40 M KCl, 0.01 M $MgCl_2$, 3.0 A_{260} units of ribosomes, 0.5 A_{260} units [^{14}C]Phe-tRNA, 0.002 M C-A(AcMet), and cytidine nucleotide at the concentrations shown. Reactions were preincubated for 10 min at 37°C; 5 min at 0°C and cytidine nucleotides used were Cp (○), CpC (●), pCp (■), pC (□). Reactions were initiated by the addition of 0.016 ml methanol. The amount of release in the absence of cytidine nucleotide is arbitrarily set at 100%. The reactions were incubated for 1 h at 0°C and terminated by the addition of 0.05 ml 3 N NaOH; products were extracted into ethylacetate after addition of 0.4 ml 5 N HCl as in [2].

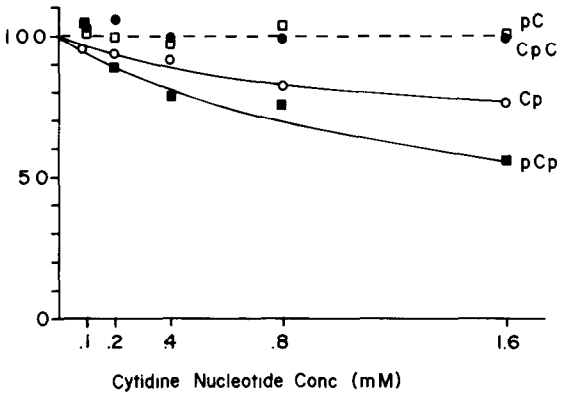

Fig.2. Effect of various cytidine nucleotides in the peptidyl transferase acceptor reaction using C-A-Lys as acceptor assays were performed as in [7]; a typical reaction mixture contained in 0.10 ml: 0.05 M Tris–HCl (pH 7.4), 0.10 M NH_4Cl, 0.01 M $MgCl_2$, 4.0 A_{260} units of ribosomes, 0.14 A_{260} units *N*-acetyl [^{14}C]Phe-tRNA, 10 μg poly(U), 1.0 × 10^{-4} M C-A-Lys, and cytidine nucleotide at the concentrations shown. Cytidine nucleotides used were pC (□), CpC (●), Cp (○), and pCp (■). Reactions were initiated by the addition of acceptor. The reaction mixtures were incubated at 37°C for 30 min and terminated by addition of 2 ml 10% trichloroacetic acid. The product was isolated by filtration and measured as in [7]. The amount of release in the absence of cytidine nucleotide is arbitrarily set at 100%.

3. Results

The effects of the cytidine nucleotides on the donor activity of C-A(AcMet) are shown in fig.1. Reactions were performed under fragment reaction conditions using [^{14}C]Phe-tRNA as an acceptor. It is therefore assumed that competition occurs at the donor site. The strongest effects are observed with pC and pCp, which are powerful inhibitors, while C-C and Cp stimulate weakly. Fig.2 illustrates similar experiments performed using C-A-Lys as the acceptor substrate. These experiments were performed in the absence of alcohol with Ac[^{14}C]Phe-tRNA as donor: competition is therefore assumed to occur at the A site. The strongest inhibition is achieved by pCp, Cp is a weaker inhibitor and C-C and pC have no effect. When a similar set of experiments using C-A-Lys as the acceptor is performed under fragment reaction conditions (fig.3), qualitatively similar results are obtained, confirming the pattern in fig.2. Thus, the different experimental conditions employed for the P and A sites are not responsible for the dif-

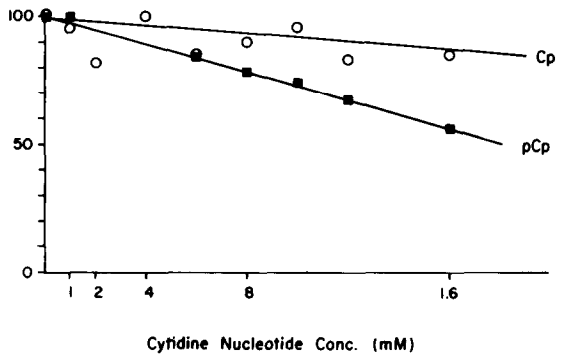

Fig.3. Effect of various cytidine nucleotides on the peptidyltransferase acceptor reaction under fragment conditions. Assays were performed as in fig.1. A typical reaction mixture contained in 0.05 ml: 0.05 M Tris–HCl (pH 7.4), 0.04 M KCl, 0.01 M $MgCl_2$, 3.0 A_{260} units of ribosomes, 0.10 A_{260} units *N*-acetyl [^{14}C]Phe-tRNA, 1.0 × 10^{-6} M C-A-Lys and cytidine nucleotide at the concentrations shown. Reactions were initiated by the addition of 0.016 ml methanol. Cytidine nucleotides used were Cp (○) and pCp (■). The reactions were terminated by addition of 2 ml 10% trichloroacetic acid and the product was isolated by filtration and measured as in [7].

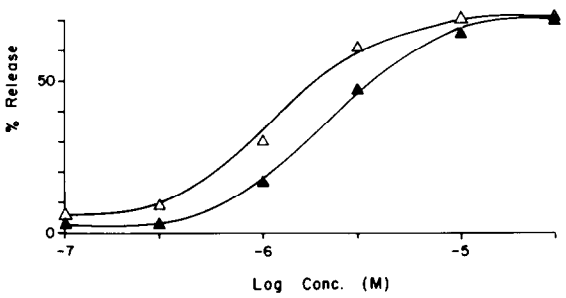

Fig.4. Acceptor activity of C-A-Lys in the presence and absence of pCp at various concentrations of C-A-Lys. The same assay was used as in fig.2, except that C-A-Lys was used at the indicated concentrations in the presence of 1.6 M pCp (▲) or in the absence of pCp (△). % release here is calculated as a percentage of the total cpm in the reaction.

fering inhibitory activities of the various cytidine nucleotides tested here. This further indicates that the effects observed under fragment reaction conditions (in the presence of methanol), are an accurate qualitative reflection of the 'natural' reaction [15]. The inhibitory effect of pCp on the transfer reaction is independent of C-A-Lys concentration (fig.4).

Table 1 illustrates the results of different approach to the investigation of the role of the cytidine residues at the 3′-terminus of tRNA. The apparent affinity constants (K_m^{app}) of the 2′(3′)-*O*-aminoacyl-derivatives of adenosine and C-A (acceptor activity) are compared. The effect of joining a cytidine 3′-phosphate residue to 2′(3′)-*O*-aminoacyladenosine is clear: a 100–500-fold increase in the acceptor activity in the peptidyltransferase reaction.

Table 1
Ratios of K_m^{app} of 2′(3′)-*O*-aminoacyl-derivatives of adenosine (A-X) and C-A (C-A-X)[a]

A-X/C-A-X	K_m^{app} A-X/C-A-X[b]
A-Phe/C-A-Phe	500
A-Lys/C-A-Lys	250
A-Met/C-A-Met	100
A-Glu/C-A-Glu	500
A-Leu/C-A-Leu	100
A-Pro/C-A-Pro	>100
A-Gly/C-A-Gly	>100

[a] Data from AcPhe-tRNA · poly(U) · 70 S ribosomes system according to [13,19].
[b] K_m^{app} is defined as the concentration of substrate at the half maximum activity [20]

4. Discussion

We report here on the role of the cytidine residues at the 3′-terminus of tRNA in the interaction of donor and acceptor substrates with their respective peptidyltransferase sites. That role can be observed either by the increased donor or acceptor activity of mononucleotide or nucleoside derivatives upon the joining of a cytidine or cytidine 3′-phosphate residue, or by the direct effect of cytidine nucleotides on the transfer reaction using either donor or acceptor substrates.

At the donor site, the donor activity of C-A(fMet) is ~30-times higher than that of pA(fMet) [4]*. The effect of joining a cytidine 3′-phosphate moiety to 2′(3′)-*O*-aminoacyladenosine as the acceptor substrate is even more dramatic, resulting in a great increase in acceptor activity. In addition, we have found that cytidine nucleotides inhibit both the acceptor and the donor activity of C-A-Lys and C-A(AcMet), respectively. Thus, pCp inhibits the transfer reaction at both sites; pC is most effective at the donor site, Cp is a more efficient inhibitor at the acceptor site. The most likely explanation of these inhibitory effects is competition between the binding of the cytidine nucleotide and the acceptor or donor substrate at the appropriate site. It logically follows that there is a locus within either the A or P site which binds the penultimate cytidylic acid residue of the acceptor or donor substrate. Other evidence points to the existence of a similar locus for binding the second cytidylic acid residue as well as at both sites [5,17]. The slight stimulation of the donor activity of C-A(AcMet) by Cp and C-C might possibly be due to interaction of these effectors with the locus for binding of second cytidylic acid residue at the donor site. This effect, however, is relatively weak and is certainly not comparable to the powerful stimulation of the donor activity of pA(fMet) by pC as reported [8]. It is apparent that the loci for binding the Cp residues within both the A and P sites are very sensitive to

* Krayesky et al. [9] investigated the donor activity of C-A(fMet). However, the synthesis of this compound by Tarussova et al. [16] could hardly lead to a product of the required purity, since the authors used a non-specific aminoacylation method on unprotected oligonucleotides, which inevitably produces many side products. Such a mixture cannot be expected to yield biochemical data open to straight-forward interpretation

relatively subtle changes in the structure of the effectors, and that the specificities of these loci are not the same for the two sites. A different structural organization within the A and P sites is probably responsible for the observed differences in the specificities of the inhibitors. The lack of stimulation of the acceptor activity of puromycin by cytidine nucleotides observed in [9] may be susceptible to the same explanation. Further, it is apparent that the second and third (Cp) residues either do not effect equally the binding of the donor or acceptor substrate, or that their loci may differ in their accessibility to effectors. The possibility of allosteric or cooperative effects within, as well as between, the A and P sites [8,18] complicates the situation and defies a simplistic explanation [10,11]. Nevertheless, these results clearly show that the binding of the Cp residues of the acceptor and donor moieties to the appropriate loci within the A and P sites plays a major role in the activities of both substrates in the peptidyltransferase reaction. Models, which do not take these facts into account [10,11], should be revised accordingly.

Acknowledgements

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