2′ (3′)-*O*-*N*-FORMYLMETHIONYL)-ADENOSINE-5′-PHOSPHATE, A NEW DONOR SUBSTRATE IN PEPTIDYL TRANSFERASE CATALYZED REACTIONS

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1. Introduction

Studies dealing with structural requirements of the binding sites of ribosomal peptidyl transferase have revealed that the substrate interaction at the acceptor site involves the terminal CpA-aminoacyl of the accepting aminoacyl-tRNA [1,2]; at the donor site, the 3′-terminal grouping of peptidyl-tRNA, the CpCpA-acylaminoacyl has been found necessary for effective interaction [3]. Monro et al. [3] have shown with a series of fragments obtained by sequential removal of 3′-mononucleotides from the 5′-end of CpApApCpCpA-fMet that CpCpA-fMet possessed the minimum structural requirement to be a donor substrate in the ribosomal fragment reaction, while CpA-fMet and A-fMet were inactive. Mercer and Symons [4] have confirmed these results with *N*-acetyl-aminoacyl-trinucleotides and -dinucleotides prepared by chemical synthesis. Recently, we have started to reinvestigate the structural requirements at the donor site and wish to report here that at high (1 mM) donor substrate concentrations 2′ (3′)-*O*-(*N*-formylmethionyl)–adenosine-5′-phosphate is a donor of the *N*-formylmethionyl residue in peptidyl transferase catalyzed reactions.

2. Materials and methods

2.1. *Preparation of ribosomes*

Ribosomes were prepared from *Escherichia coli* B as described elsewhere [5]. The ribosomes were activated by preincubation at 40°C according to Miskin et al. [6].

2.2. *Preparation of substrates*

The terminal fragments CpApCpCpA-[^{3}H] Phe and CpApCpCpA-[^{3}H] Leu were prepared as described earlier [3]. The 2′ (3′)-*O*-(*N*-formylmethionyl)-adenosine-5′-phosphate was prepared by the method for synthesis of *N*-acyl-aminoacyl-nucleoside-5′-phosphates developed by Gottikh et al. [7].

2.3. *Transfer assay*

The assay is essentially that of Monro and Marcker [8]: reaction mixtures contained (prior to methanol addition) 0.05 M Tris–HCl buffer pH 7.4, 0.4 M KCl, 0.02 M magnesium acetate, ribosomes (440 μg), CACCA-[^{3}H] Phe (150–200 pmoles) and pA-fMet at the concentration shown for the individual experiments. The reaction was initiated by addition of methanol (30–50% of the total volume) and incubation was at 0°C for 60 min. The reaction was terminated by addition of 50 μl of 3 N NaOH to hydrolyze the ester bond between oligonucleotide and peptide or unreacted acceptor. After incubation for 30 min at 36°C, 400 μl of 5 N HCl was added and the fMetPhe formed was extracted from this acid solution with 3 ml of ethyl acetate and counted. The blank value was obtained by omitting pA-fMet.

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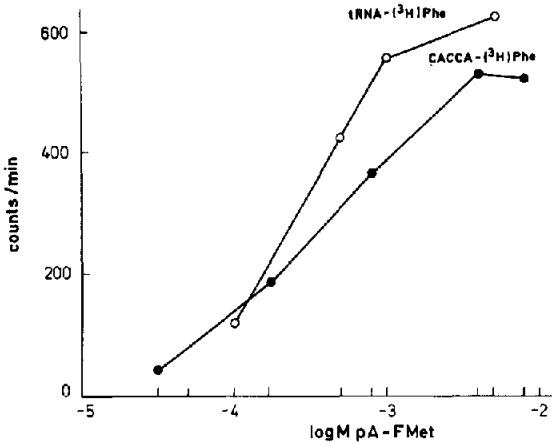

Fig. 1. Reaction of pA-fMet with CpApCpCpA-[^{3}H]Phe and [^{3}H]Phe-tRNA. Conditions of assay are described in Materials and methods.

2.4. *Identification of reaction products*

The peptide was split from the nucleotide moiety by mild alkaline hydrolysis under the same conditions as in the transfer assay, and after acidification and extraction with ethyl acetate, fMetPhe was separated by high-voltage electrophoresis on paper [9].

3. Results

3.1. *Donor activity of chemically synthesized pA-fMet*

We tested the donor activity of pA-fMet, which represents the terminal sequence of the natural initiator fMet-tRNA under conditions of the fragment reaction [8]. As acceptor we used CpApCpCpA-[^{3}H]Phe labelled in the phenylalanine moiety. Results summarized in fig. 1 show that the transfer reaction occurs at high concentration of the donor substrate (1 mM), which is about 500 times that tested by Mercer and Symons (2 μM) [4], and about 10 000 times that tested by Monro et al. (10 nM) [3]. Similar results were obtained with [^{3}H]Phe-tRNA as acceptor (fig. 1).

3.2. *Identification of the product of the transfer reaction*

The dipeptide fMet[^{3}H]Phe was identified as product of the transfer reaction of pA-fMet as donor with CpApCpCpA-[^{3}H]Phe as acceptor substrate after cleavage from the expected CpApCpCpA-fMet[^{3}H]Phe.

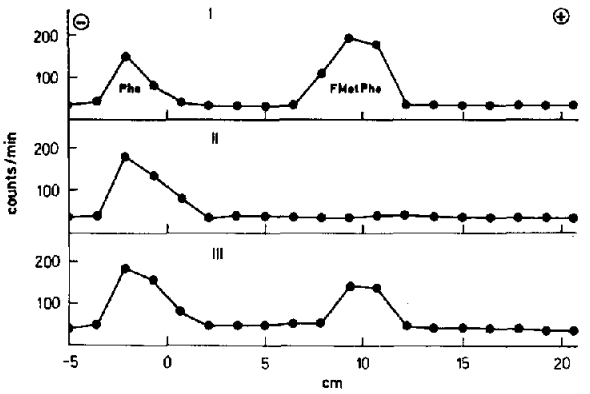

Fig. 2. Paper electrophoretic analysis of products formed by transfer of the fMet residue from pA-fMet to CpApCpCpA-Phe. The reaction products were separated after alkaline hydrolysis by high-voltage electrophoresis at pH 3.5. Phe, I) fMet-Phe, reference samples; II) CpApCpCpA-[^{3}H]Phe after alkaline hydrolysis; III) products of the reaction CpApCpCpA-[^{3}H]Phe+pA-fMet after alkaline hydrolysis.

Only two peaks of radioactivity were detected on the chromatogram after high voltage electrophoresis, one corresponding to phenylalanine, the other to fMetPhe prepared by chemical synthesis (fig. 2).

3.3. *The dependence of the transfer reaction on ribosomes*

The transfer of the fMet-residue from pA-fMet to CpApCpCpA-[^{3}H]Phe is completely dependent on the presence of ribosomes and of methanol (table 1). These observations show that a non-enzymic, peptidyl transferase independent acylation of the acceptor substrate does not occur.

Table 1
Reaction of pA-fMet with CpApCpCpA-[^{3}H]Phe.

	Ethyl acetate soluble cpm
Complete system	1318
Ribosomes omitted	474
pA-fMet omitted	464
Methanol omitted	530

Conditions of assay are described in Materials and methods.

Table 2
The effect of chloramphenicol on donor activity of pA-fMet.

Chloramphenicol concn. (μM)	Acceptor substrate			
	[^{3}H]Phe-tRNA (cpm)	(%)	CpApCpCpA-[^{3}H]Phe (cpm)	(%)
–	556	100	700	100
100	56	10	76	11
50	156	28	–	–

Donor activity of pA-fMet was examined under conditions described in Materials and methods.

Table 3
Dependence of the reaction pA-fMet with CpApCpCpA-[^{3}H]Phe on the concentration of Mg^{2+} ions.

Concn. Mg^{2+} (mM)	Ethyl acetate soluble cpm
3	18
10	120
30	400
60	463

Conditions of assay are described in Materials and methods; 50 pmoles of CpApCpCpA-[^{3}H]Phe were used as acceptor substrate.

3.4. *The effect of chloramphenicol*

Chloramphenicol is known to inhibit peptidyl transferase [10]. As shown in table 2 the transfer of fMet to CpApCpCpA-[^{3}H]Phe and also to [^{3}H]Phe-tRNA is inhibited by chloramphenicol.

3.5. *The effect of Mg^{2+} and of Ca^{2+} ions on the transfer reaction*

Mg^{2+} ions are required for the transfer of the fMet residue from pA-fMet (table 3). In their absence no reaction takes place. In contrast to Mg^{2+} ions Ca^{2+} ions were inert.

3.6. *Transfer reaction with further substrates*

In addition to CpApCpCpA-[^{3}H]Phe and [^{3}H]Phe-tRNA we examined the transfer of fMet from pA-fMet to CpApCpCpA-[^{3}H]Leu and to [^{3}H]puromycin. Fig. 3 shows the time course of the transfer. Puromycin has a similar acceptor activity as the Phe-pentanucleotide while the Leu-pentanucleotide is less active as acceptor.

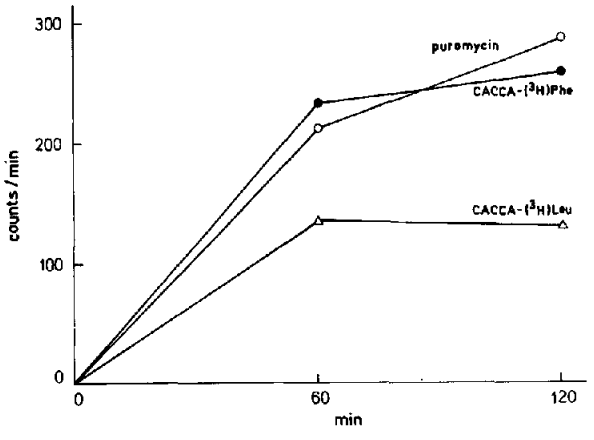

Fig. 3. Time course of pA-fMet (3^4 mM) with [^{3}H]puromycin, CpApCpCpA-[^{3}H]Phe and CpApCpCpA-[^{3}H]Leu. Conditions were as described in Materials and methods.

4. Discussion

Monro et al. [3] and Mercer and Symons [4] have shown that the minimal sequence CpCpA-acylaminoacyl is required for donor substrate activity in peptidyl transferase catalyzed reaction.

Our observation indicates that these restrictive requirements are valid only under certain conditions. We have found a small but significant transfer of the fMet-residue from pA-fMet, which does not meet the above restriction, when pA-fMet was used at rather high concentration (1 mM). Under these conditions 2–5% of added acceptor substrate was acylated with fMet, i.e. about 6–16 pmoles. This corresponds to acylation of 12–30% of acceptor substrate attached to the acceptor site.

The observed transfer of the fMet residue from pA-fMet to acceptor substrate has all characteristics expected of a reaction catalyzed by peptidyl transferase. The fMet-residue is transferred on the α-amino group of the aminoacyl residue in the acceptor substrate (fig. 2). The reaction is inhibited by chloramphenicol, a specific inhibitor of peptidyl transferase, to the same extent as reactions with the more complex substrates (table 2). Mg^{2+} ions are required for the transfer of the fMet-residue from pA-fMet, while

Ca^{2+} ions are inactive similarly as with more complex substrates (table 3) [11]. The rate of the transfer reaction depends on the nature of the aminoacyl residue in the acceptor substrate in the same way as was found for acPhe-tRNA or acLeu pentanucleotide donors [12]. All these characteristics indicate that the transfer of the fMet-residue from pA-fMet to acceptor substrate is catalyzed by peptidyl transferase and that compounds of the pA-fMet type are up to the present the simplest donor substrates for this enzyme.

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