THE DINUCLEOSIDE MONOPHOSPHATE, CpC, IS A MODEL ACCEPTOR SUBSTRATE FOR RABBIT-LIVER tRNA NUCLEOTIDYLTRANSFERASE

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1. Introduction

The enzyme tRNA nucleotidyltransferase catalyzes the incorporation of AMP and CMP residues into the 3′-terminal –C–C–A sequence found on all tRNA molecules [1]. In the presence of the appropriate ribonucleoside triphosphate(s), the homogeneous enzyme from rabbit liver is able to repair correctly tRNA molecules missing all, or part, of this defined sequence [2]. Since the purified enzyme contains no nucleotide material which could act as a template for this process [3], we proposed several years ago that synthesis of the –C–C–A sequence required a specific arrangement of active sites recognizing the terminal residues [4]. We suggested that tRNA nucleotidyltransferase would contain two CTP-binding sites which would also recognize the terminal –C–C residues, and in addition, one ATP-site, and that these three sub-sites would be arranged in tandem. A similar model was also proposed by Rether et al. [5]. These models were consistent with a variety of kinetic experiments carried out with purified enzymes [1,4,5].

In order to obtain further evidence in support of a three-site model, we have tried to devise a model system for tRNA nucleotidyltransferase action which would not require a complete tRNA molecule, but only the terminal residues as acceptors for AMP-incorporation. In this paper we show that the dinucleoside monophosphate, CpC, is a substrate for the synthesis of CpCpA, and that the reaction has the same ribonucleoside triphosphate specificity as tRNA–C–C. The reaction can be conveniently monitored by measurement of the release of [^{32}P]pyrophosphate (PP_i) from [γ-^{32}P]ATP. Development of this assay opens up the possibility of using other defined acceptors to dissect the active site of tRNA nucleotidyltransferase.

This paper is number 20 in the series: Reactions at the 3′-Terminus of Transfer Ribonucleic Acid. The previous paper in the series is reference [10].

2. Materials and methods

[γ-^{32}P]ATP was purchased from ICN Pharmaceuticals and purified on Dowex-1-Cl to remove Norit non-adsorbable counts. [^{14}C]ATP was obtained from Schwarz/Mann. CpC was purchased from Sigma Chemical Co. and CpCpA from Miles Biochemicals. Rabbit-liver tRNA nucleotidyltransferase, purified through step 8 (about 500 units/mg protein [4]), was used for all the experiments reported here. Rabbit liver tRNA–C–C was prepared as previously described [6].

Enzyme activity was measured by pyrophosphate release. This assay measures the formation of [^{32}P]PP_i from [γ-^{32}P]ATP upon incorporation of the AMP moiety. [^{32}P]PP_i was determined as radioactivity not bound to Norit. Reaction mixtures contained in 0.1 ml: 50 mM glycine–NaOH, pH 9.4; 10 mM $MgCl_2$; 0.5 mM [γ-^{32}P]ATP (about 2500–3000 cpm/nmol); tRNA–C–C or dinucleoside monophosphate at the indicated concentration; and enzyme. After incubation at 37°C the reaction was terminated by the addition of 0.3 ml 0.5 M perchloric acid containing 1 mM sodium pyrophosphate and 0.1 ml Norit suspension (168 mg/ml).

The suspension was agitated on a vortex mixer, then centrifuged for 5 min at 10 000 rev/min. An aliquot (0.3 ml) of the supernatant fluid was counted in Triton–toluene scintillation fluid. Blanks in the absence of enzyme have been subtracted to obtain the data presented.

3. Results

In the absence of a suitable acceptor for AMP, no detectable [^{32}P]PP_i was released from ATP. We have not obtained any evidence for even a single turnover of the enzyme in the absence of an acceptor, suggesting that enzyme–AMP is not an intermediate in this reaction. However, in the presence of the dinucleoside monophosphate, CpC, pyrophosphate formation proceeded linearly for at least one hour (fig.1), suggesting that CpC could act as an acceptor of AMP-residues.

Variation of the CpC concentration in the reaction mixture indicated that this substrate followed normal saturation kinetics (fig.2). The apparent K_m for CpC calculated from this data was 5 mM and the V_{max} was 16 μmol/h/ml enzyme under these conditions. This compared with values for tRNA–C–C of 4 μM and 250 μmol/h/ml enzyme, respectively (table 1). Thus, although CpC is a substrate for tRNA nucleotidyltransferase, it has an apparent K_m about three orders of magnitude greater than tRNA–C–C and a V_{max} less than one-tenth that of the macromolecular substrate.

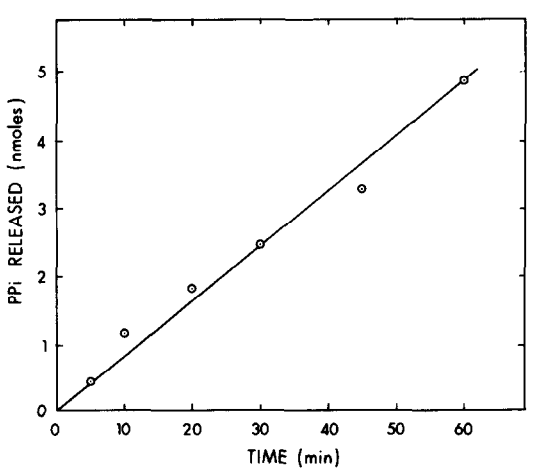

Fig.1. Time-course of [^{32}P]PP_i release from [γ-^{32}P]ATP in the presence of CpC. Reaction mixtures were as described in Materials and methods with 1 mM CpC and 0.6 unit tRNA nucleotidyltransferase. Samples were removed at the times indicated and the Norit non-adsorbable radioactivity determined.

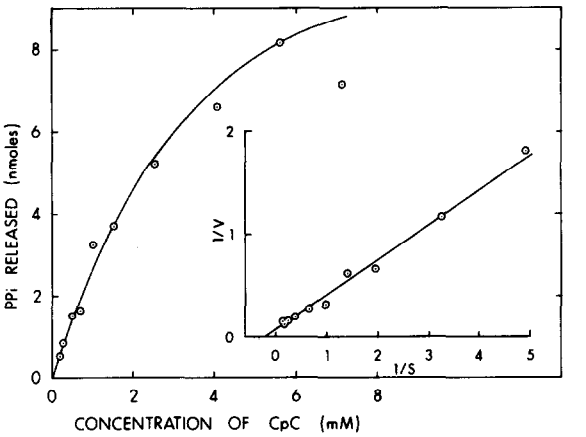

Fig.2. Effect of CpC concentration on the rate of [^{32}P]PP_i release from [^{32}P]ATP. Reaction mixtures were as described in Materials and methods with 0.6 unit enzyme and CpC varying from 0.2–7 mM. Incubation were for 30 min at 37°C.

In order to confirm that the expected product, CpCpA, was synthesized when CpC was the acceptor substrate, aliquots of reaction mixtures containing [^{14}C]ATP were subjected to high-voltage electrophoresis. As shown in fig.3, a peak of radioactivity was found which migrated with an authentic CpCpA marker. No other radioactive products were found. Alkaline hydrolysis of the radioactive product after electrophoresis [7] released all the radioactivity (> 95%) as [^{14}C]adenosine. These results indicated that only a single AMP-residue was added to CpC, and suggested

Table 1
Comparison of kinetic constants for tRNA–C–C and CpC

Acceptor substrate	Apparent K_m (μM)	Apparent V_{max} (μmol/h)
tRNA–C–C	4	250
CpC	5000	16

The K_m and V_{max} for CpC were determined from the data in fig.2. The constants for tRNA–C–C were determined in a parallel experiment using 0.03 unit enzyme and incubation for 2 min at 37°C. The V_{max}-values have been normalized to 1 ml enzyme.

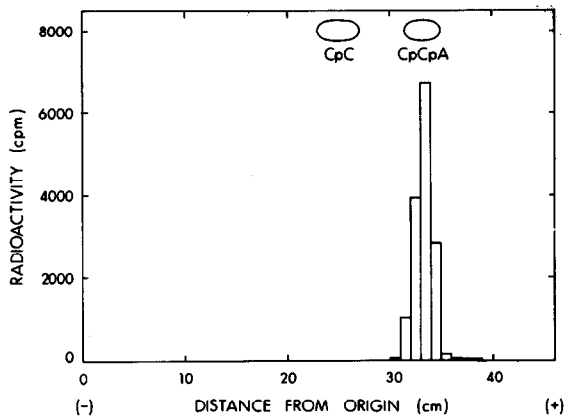

Fig.3. High-voltage electrophoresis of the product of the reaction of ATP and CpC. Reaction mixtures were as described in Materials and methods with 5 mM CpC, 0.5 mM [^{14}C]ATP (6000 cpm/nmol) and 0.9 unit enzyme. After 1 hour at 37°C, an aliquot of the reaction mixture was removed and applied to Whatman 3 MM paper. Electrophoresis was carried out in 0.02 M Na-citrate buffer, pH 5.0, at 50 V/cm for 2 h with a picric acid standard. Marker CpC and CpCpA migrated as indicated. A sample without enzyme was also subjected to electrophoresis and has been subtracted to obtain the data presented. The [^{14}C]ATP-peak was at 45 cm.

that the dinucleoside monophosphate acts as an analogue of the 3′-terminus of tRNA–C–C.

Further support for this suggestion came from studies with the three other ribonucleoside triphosphates (table 2). As with the macromolecular substrate, tRNA–C–C, CTP could act as a donor for CMP-incorporation into CpC, although more poorly than ATP, whereas UTP and GTP were inactive as donors [4,8]. From these results, and preliminary experiments with other dinucleoside monophosphate, we conclude that CpC is a model substrate for the terminal nucleotides of tRNA–C–C.

Table 2
Comparison of nucleotide incorporation into CpC

Nucleoside triphosphate	Nucleotide incorporated (nmol)
ATP	8.1
CTP	3.1
UTP	< 0.1
GTP	< 0.1

Reaction mixtures were as described in Materials and methods with 5 mM CpC, 0.5 mM of the indicated [^{14}C]nucleoside triphosphate (5000–6000 cpm/nmol) and 0.9 unit enzyme. After 1 h at 37°C aliquots were removed and subjected to high voltage electrophoresis in 0.015 M citrate buffer, pH 4.2, at 50 V/cm for 2 h. Strips of 1.5 cm were cut out and counted, and the total radioactivity incorporated was determined. Blank samples without enzyme were also subjected to electrophoresis and have been subtracted to obtain these values.

4. Discussion

The data presented here indicate that a substrate as small as CpC is sufficient for tRNA nucleotidyltransferase catalysis to occur. However, since the K_m- and V_{max}-values are very different compared to tRNA–C–C, the rest of the tRNA molecule must also play a role in stabilizing the interaction between the 3′-terminal residues and the enzyme, as well as promoting more efficient catalysis. These results suggest experiments to determine the structural features on tRNA which are important for efficient tRNA nucleotidyltransferase action. Thus, elongation of the structure of CpC and addition of complementary oligonucleotides, in a stepwise manner, should permit elucidation of the parts of the tRNA molecule most important for binding and catalysis. Zachao's group has previously shown that half and three-quarter molecules of tRNA could act as acceptors of AMP and CMP [9].

The finding that CpC acts as a model for the 3′-terminus of tRNA–C–C also provides a method for distinguishing between effects on tRNA structure and on the reaction itself. For example, we previously reported [6] that polyamines stimulate tRNA nucleotidyltransferase, and we suggested that the effect was due to alteration of the conformation of tRNA. The use of CpC, which does not contain the remainder of the tRNA molecule should permit a test of this hypothesis.

Finally, the ability of a dinucleoside monophosphate to act as an acceptor for AMP will enable us to dissect the active site of tRNA nucleotidyltransferase. In fact, preliminary experiments with the six other dinucleoside monophosphates containing cytidine in one of the two positions have indicated that only CpC is an effective acceptor of AMP. These results suggest that the enzyme specifically recognizes cytidine residues in each of the two terminal positions, and supports the three-site model for tRNA nucleotidyltransferase catalysis [4].

Acknowledgements

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References

[1] Deutscher, M. P. (1973) Progr. Nucleic Acid Res. Mol. Biol. 13, 51–92.
[2] Deutscher, M. P. (1972) J. Biol. Chem. 247, 469–480.
[3] Deutscher, M. P. (1972) J. Biol. Chem. 247, 450–458.
[4] Deutscher, M. P. (1972) J. Biol. Chem. 247, 459–468.
[5] Rether, B., Gangloff, J. and Ebel, J-P. (1974) Eur. J. Biochem. 50, 289–295.
[6] Evans, J. A. and Deutscher, M. P. (1976) J. Biol. Chem. 251, 6646–6652.
[7] Deutscher, M. P. (1973) J. Biol. Chem. 248, 3116–3121.
[8] Deutscher, M. P. (1973) J. Biol. Chem. 248, 3108–3115.
[9] Overath, H., Fittler, F., Harbers, K., Thiebe, R. and Zachav, H. G. (1970) FEBS Lett. 11, 289–294.
[10] Deutscher, M. P., Foulds, J. and Setlow, P. (1977) submitted.