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Anticodon-dependent aminoacylation of RNA minisubstrate by lysyl-tRNA synthetase

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Specific inhibition of mammalian lysyl-tRNA synthetase by polyU is shown. Inhibition of the enzyme is dependent on the length of the oligonucleotide, since oligoU molecules with a length of less than 8 residues do not inhibit the aminoacylation, whilst the effect of oligoU molecules with a length of about 30 residues is the same as that of polyU. Inhibition is a result of recognition by the enzyme of the $tRNA^{Lys}$ anticodon sequence (UUU) coded by polyU. Aminoacylation of the oligoU molecule with attached CCA sequence ($G(U)_{20}$–CCA) by yeast and mammalian lysyl-tRNA synthetases is demonstrated.

Lysyl-tRNA synthetase; RNA-minisubstrate; tRNA recognition

1. INTRODUCTION

Aminoacylation of tRNAs with cognate amino acids by aminoacyl-tRNA synthetases is an essential step in the translation of the genetic code. Identity of tRNAs is determined by a limited set of nucleotides, and the anticodon has been found to be the major identifying element in many tRNAs [1]. In previous studies of non-specific interaction of eukaryotic aminoacyl-tRNA synthetases with polyanions [2], unusually high affinity of mammalian lysyl-tRNA synthetase for polyU was observed [3]. Together with other observations that anticodon of $tRNA^{Lys}$ ($^{U}_{C}UU$) was important for the recognition by lysyl-tRNA synthetase [4,5], our results raised the possibility that the observed high affinity of the enzyme to polyU results from recognition of the anticodon sequence (UUU) within the polyU molecule. Here we present a detailed study of the interaction of lysyl-tRNA synthetase with polyU and show that quite short oligoU molecules, with CCA attached to their 3′ end, can serve as substrates for this enzyme.

2. MATERIALS AND METHODS

The multi-enzyme aminoacyl-tRNA synthetase complex purified from rabbit liver [6] was used as the source of lysyl- and arginyl-tRNA synthetase activity. Yeast lysyl-tRNA synthetase was the generous gift of Dr. P. Kerjan (Laboratoire d'Enzymologie, Gif-sur-Yvette). Synthetic DNA fragments corresponding to the oligonucleotide gene and comprising the consensus T7 promoter region (positions −17 to +1) were synthesized and cloned by *Hin*dIII,*Bam*HI sites in pUC18 plasmid. The sequence of the gene was verified by sequencing. The oligoU–CCA ($G(U)_{20}$-CCA) molecule was synthesized in a transcription reaction [7] using T7 RNA polymerase and a double-stranded DNA template obtained by linearization of the cloned DNA. Transcription reactions were performed in 300 μl of 40 mM Tris-HCl buffer (pH 8.1), containing 22 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.05 mg/ml bovine serum albumin, 5 mM of each NTP, 50 μg linearized DNA, 50 U RNAsin and 800 U of T7 RNA polymerase. The reaction mixture was incubated for 2 h at 42°C and the transcript, after deproteinization and precipitation with 3 vols. of acetone containing 2.5% $NaClO_4$, was purified by MonoQ chromatography [8]. Concentration of oligoU–CCA was determined spectrophotometrically, taking into account the extinction coefficient of uridine. OligoC–CCA was prepared by ligation of pAp to the C_{23} molecule with RNA ligase, followed by phosphatase treatment [9].

Aminoacylation of tRNAs was performed as described elsewhere [6]. Aminoacylation of oligoU–CCA was carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 25 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2.5 μM [^{3}H]lysine (Amersham), 1 mM dithiothreitol and 1 U/ml of inorganic pyrophosphatase. Reaction mixtures were incubated at 37°C and the reaction was terminated by addition of 5 μg of polyA to 50 μl samples. Samples were additionally incubated for 2 min and further precipitated by cold 10% trichloroacetic acid. The presence of polyA is required for the complete precipitation of oligoU–CCA molecules. The reaction stops immediately after polyA is added. The aminoacylation test using DE-81 paper filters was done as described by Frugier et al. [10]. Electrophoresis of aminoacylated oligoU–CCA molecules at acid pH in 20% polyacrylamide gels was performed as described by Martinis and Schimmel [11].

OligoU molecules of different lengths were obtained by alkaline hydrolysis of polyU followed by MonoQ chromatography [12].

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3. RESULTS

The specificity of lysyl-tRNA synthetase inhibition by polyU is demonstrated in Fig. 1. For comparison, inhibition curves of both lysyl- and arginyl-tRNA synthetases by polyanionic polysaccharide heparin, which is known to bind non-specifically to eukaryotic aminoacyl-tRNA synthetases [2], are presented. As clearly shown, polyU practically completely inhibited lysyl-

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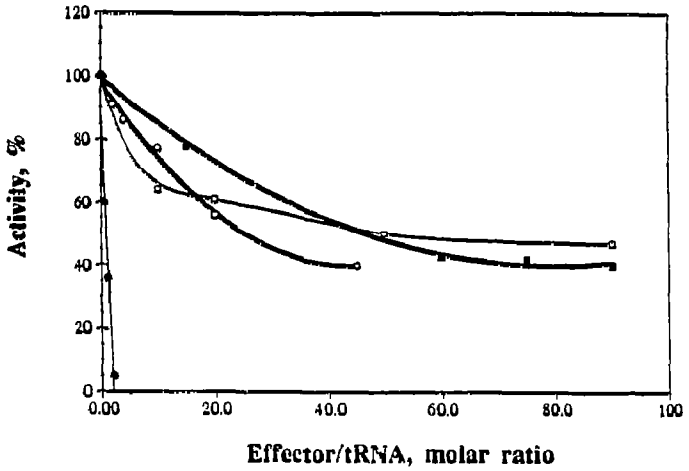

Fig. 1. Inhibition by polyU and heparin of the aminoacylation of tRNA by lysyl- and arginyl-tRNA synthetases. Lysyl-tRNA synthetase + polyU (●) or heparin (○); arginyl-tRNA synthetase + polyU (□) or heparin (■). Concentrations of $tRNA^{Lys}$ and $tRNA^{Arg}$ were 3×10^{-7}M and 4×10^{-7} M, respectively.

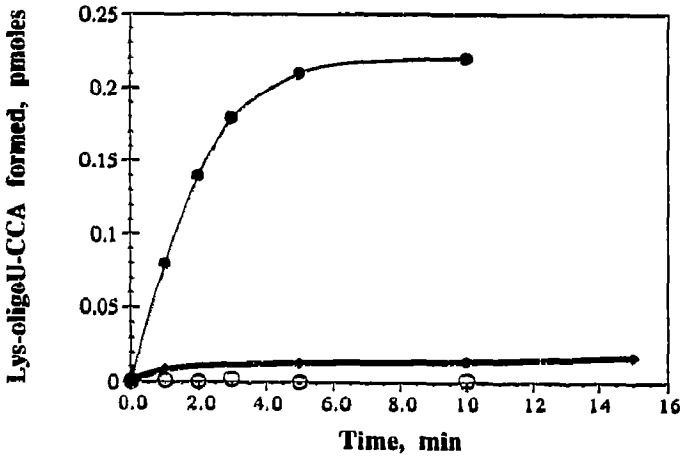

Fig. 3. Kinetics of oligoU-CCA aminoacylation by yeast (●) and mammalian (◆) enzymes. Aminoacylation of oligoC-CCA by yeast lysyl-tRNA synthetase (○). Background has been subtracted from the values presented. Concentrations of oligoU-CCA and oligoC-CCA are 200 nM. Concentrations of the enzymes are 50 nM.

tRNA synthetase activity at a molar ratio of 1:1 with tRNA, while the effect of polyU on arginyl-tRNA synthetase is of the same order as that of heparin on both enzymes. K_i values for inhibition of lysyl-tRNA synthetase by polyU and heparin are 4.2×10^{-8} M and 1.5×10^{-5} M, respectively. Inhibition of lysyl-tRNA synthetase by polyU appeared to be specific, because not only arginyl-, but also mammalian phenylalanyl- and valyl-tRNA synthetases were inhibited by polyU to a much lesser extent than lysyl-tRNA synthetase ([3], and A.W., unpublished results).

To evaluate the length of polyU required to inhibit lysyl-tRNA synthetase, a set of oligoU molecules of different lengths was tested (Fig. 2). The concentration of oligoU was normalized to correspond to 10 μM of monomeric uridine in the assay. OligoU molecules with a length of less than 8 residues had no effect on enzyme activity at the concentration used, while molecules with a length of about 30 residues inhibited aminoacylation with the same efficiency as polyU.

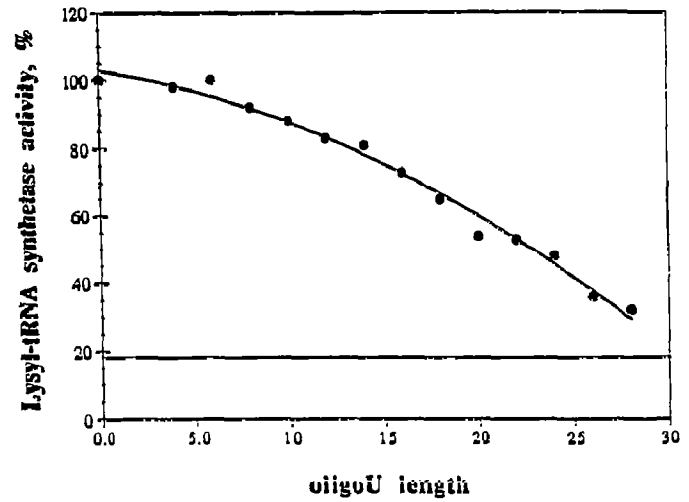

Fig. 2. Effect of oligoU chain length on lysyl-tRNA synthetase activity. The solid line presents the level of inhibition by polyU at the same concentration (10 μM of monomeric U).

To test the aminoacylation of oligoU molecules with CCA attached to their 3′ end, cloning of the artificial gene carrying T7 RNA polymerase promoter followed by in vitro transcription were used to synthesize the $G(U)_{20}$–CCA molecule. Because of T7 polymerase requirements, it starts with the first G. To detect the oligoU–CCA aminoacylation, addition of polyA to the reaction mixtures was required, because quantitative precipitation of oligoU–CCA molecules by trichloroacetic acid could not be achieved. Alternatively, aminoacylation could be assayed by adsorption of the oligoU–CCA molecules on DEAE-81 paper filters, but the recovery of aminoacylated oligoU–CCA molecules by this procedure was lower (data not shown), probably due to the hydrolysis of aminoacylated molecules by DEAE groups. Both mammalian and yeast lysyl-tRNA synthetases were shown to aminoacylate oligoU–CCA molecules (Fig. 3). No aminoacylation of $(C)_{21}$–CCA molecules by the yeast enzyme was observed under the same conditions. Activities of both enzymes were very low compared to their activity in tRNA aminoacylation, and the levels of oligoU–CCA aminoacylation did not exceed 3–4%. Attachment of [^{3}H]lysine to oligoU–CCA was also confirmed by gel electrophoresis of the aminoacylated molecules, followed by fluorography (data not shown).

4. DISCUSSION

The importance of the anticodon sequence for $tRNA^{Lys}$ identity was demonstrated by various bio-

chemical studies [13–15] and by in vivo studies using suppressor tRNAs [4]. Finally, a recent in vitro study of the aminoacylation of $tRNA^{Lys}$ transcripts by *E. coli* lysyl-tRNA synthetase clearly established the crucial role of uridine in the anticodon of $tRNA^{Lys}$ for the recognition by the enzyme [5]. Our results demonstrate that mammalian lysyl-tRNA synthetase is strongly and specifically inhibited by polyU. The tight binding of yeast lysyl-tRNA synthetase to polyU was also observed earlier [16]. This unusual affinity could be explained by the recognition by the enzyme of the anticodon sequence (UUU) coded by polyU, which led to the high-affinity binding of this RNA to the enzyme. The length dependence of the inhibitory effect of oligoU allows the suggestion that effective binding of oligoU to the enzyme requires not only specific interactions with the anticodon sequence, but also some additional interactions with the polynucleotide chain.

Based on the finding that comparatively short oligoU molecules still have significant affinity for the enzyme, the $G(U)_{20}$–CCA molecule was tested as a substrate of lysyl-tRNA-synthetase, and aminoacylation activity of this enzyme was shown. Detected activity of the mammalian enzyme is more than three-orders lower than in aminoacylation of corresponding tRNAs, and, although it did not allow the accurate determination of kinetic constants, the estimated K_m value was in the order of 10^{-7} M. The yeast enzyme displayed comparatively higher activity and the K_m value for the oligoU–CCA aminoacylation was determined to be 4.1×10^{-7} M. These results indicate the remarkably high affinity of the oligoU–CCA molecule to lysyl-tRNA synthetases. Aminoacylation of oligoU–CCA molecules is specific to the RNA substrate since no aminoacylation of oligoC–CCA molecules of the same length was observed.

Aminoacylation of small artificial RNA substrates was shown for alanyl-, histidyl-, methionyl-, glycyl- and valyl-tRNA synthetases [10,17]. In all these cases minisubstrates resembled the acceptor stem of the corresponding tRNAs, and aminoacylation was ensured by the presence of some of the tRNA identifying elements in the minisubstrate molecules. Since the oligoU–CCA molecule has nothing in common with tRNAs in the vicinity of the acceptor end, it appears that only the anticodon sequence within this molecule is responsible for the recognition and aminoacylation by lysyl-tRNA synthetase. To our knowledge, this is the first example in which aminoacylation of the tRNA minisubstrate is ensured only by the anticodon sequence, distantly located from CCA-end as the identifying element. Moreover oligoU–CCA aminoacylation is the first example of a single-stranded RNA serving as substrate for an aminoacyl-tRNA synthetase.

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