T4 RNA LIGASE: SUBSTRATE CHAIN LENGTH REQUIREMENTS

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1. Introduction

RNA ligase was discovered by Silber et al. [1] in extracts of *E. coli* cells infected with T4 bacteriophage. The enzyme catalyzes the conversion of linear polyribonucleotides to a circular form. In this reaction an intramolecular covalent linkage is formed between the 5′-phosphate end and the 3′-hydroxyl terminus. Such an intramolecular joining reaction is thought to be constrained by the probabilities of juxtaposition of the reactive termini and by the strain induced upon ring closure [2]. Thus, the rate of this reaction is expected to vary with chain length and composition of the reacting polyribonucleotides. Knowledge of these factors may serve as a measure of the conformation of various polyribonucleotides and indicate the in vivo role of RNA ligase.

In the present studies we examined a series of oligo (pA) with increasing chain length as substrates for the enzyme. It was found that $(pA)_8$ is the shortest oligonucleotide in this series that can be circularized by the enzyme.

2. Materials and methods

[γ-^{32}P] ATP was prepared according to Avron [3]. Oligo(pA) fractions were prepared by mild alkaline hydrolysis of poly(A) with 7N NH_4OH at 37°C, the incubation time varied according to the desired average chain length of the products. To cleave any cyclic phosphate groups, the various oligo(Ap)fractions were incubated with 0.1 N HCl for 1 hr at 23°C and then brought to pH 8.0 with Tris base. The 2′- or 3′-phosphate groups were then removed by passing the oligo(Ap)fraction through a column of alkaline phosphatase covalently bound to Sepharose 4B at 37°C [4]. The free 5′-hydroxyl termini of the various oligo(A) fractions were labeled with ^{32}P by the use of polynucleotide kinase as described by Silber et al. [1]. Oligo(pA) preparations with a relative short average chain length were subjected to DEAE-cellulose column chromatography, using an NH_4HCO_3-adenosine gradient (fig. 1). The addition of adenosine to the eluting solution was found to decrease the interaction of the oligonucleotide bases with the resin, and yielded oligo(pA) fractions with homogenous chain length. To remove the adenosine, the individual oligonucleotide fractions were diluted 5-fold with water and adsorbed on 1 X 2 cm DEAE-cellulose columns that were equilibrated with 0.01 M NH_4HCO_3. The columns were washed first with 10 ml of 0.01 M NH_4CO_3 followed by 10 ml 0.01 M NH_4CO_3 1 M buffer. The 1 M effluent was lyophilized and the residue dissolved in water. Unchromatographed hydrolyzates containing aligo(pA) of an average chain length of 15 or above were dialyzed extensively against 0.1 M NaCl and then against water. The chain length of the various oligo(pA) fractions was determined by electrophoresis on 16% polyacrylamide – 7 M urea gels or on 2.5% polyacrylamide – Agarose gels, using appropriate markers of known chain length. Polynucleotide kinase was prepared from *E. coli* MRE 600 cells infected with T4 phage [5]. RNA ligase was isolated from *E. coli* MRE 600 cells infected with T4 phage and purified according to the procedure of Silber et al. [1] through the Sephadex G-75 step. A linear gradient of KCl ranging between 0.2 – 0.7 M was used to elute the enzyme from the DEAE-cellulose column. RNA ligase was assayed in a reaction mixture (0.025 ml) containing [5′-^{32}P] oligo(pA) (10^8 – 10^9 cpm/μmole); 50 mM Tris–HCl,

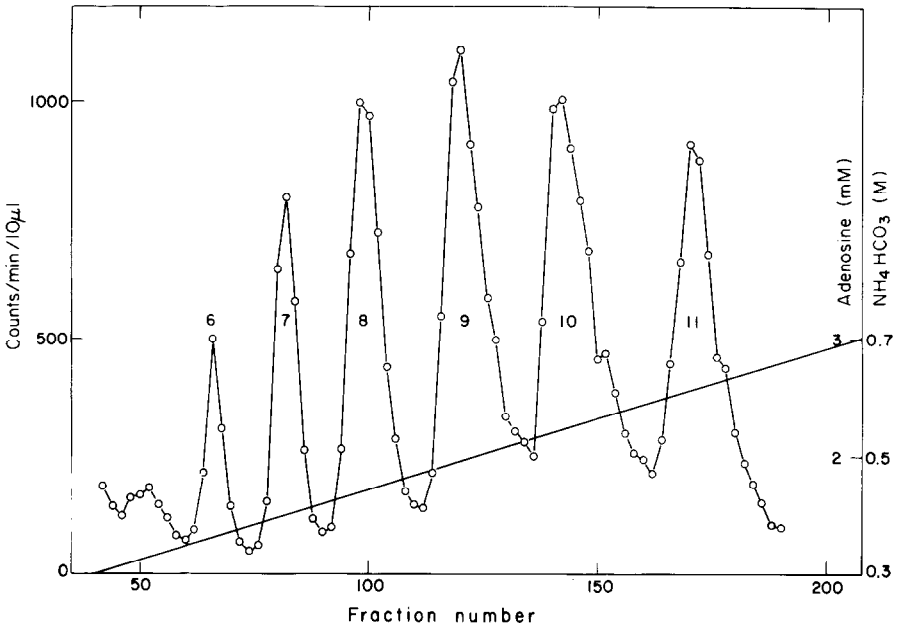

Fig. 1. Fractionation of [5′-^{32}P]oligoadenylates by DEAE-cellulose column chromatography using NH_4HCO_3-adenosine gradient. Poly (A) was incubated in 7N NH_4OH for 10 hr at 37°C and the resulting oligo(Ap) mixture was dephosphorylated as described in Materials and methods. 50 A_{260} units of this preparation were fractionated on a 1 × 5 cm DEAE-cellulose column by stepwise elution with NH_4HCO_3. The fraction emerging between 0.4–0.6 M salt concentrations was phosphorylated with polynucleotide kinase in a reaction mixture (5.0 ml) containing 18 A_{260} oligo(A), 50 mM Tris–HCl, pH 7.6; 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM [γ-^{32}P] ATP (10^9 cpm/mole), 10 μg/ml bovine serum albumin and 200 units of polynucleotide kinase. After 90 min at 37°C, 200 units enzyme were added in 2 ml of 0.05M Tris–HCl pH 7.5, 0.01 M β-mercaptoethanol and incubation continued for 60 min, after which the mixture was extracted with aqueous phenol. The aqueous phase was supplemented with adenosine (1 mM) and applied to a 0.5 × 40 cm DEAE-cellulose (Whatman DE-52) column, equilibrated with 0.01 M NH_4HCO_3 containing 1 mM adenosine. The column was washed with 50 ml of 0.3 M NH_4HCO_3, 1 mM adenosine followed with a linear gradient consisting of 200 ml each of 0.3 M NH_4HCO_3, 1 mM adenosine and 0.7 M NH_4HCO_3, 3 mM adenosine. Fractions of 2.5 ml were collected at 6 ml/hr.

pH 7.5; 10 mM $MgCl_2$; 0.1 mM ATP; 20 μg/ml bovine albumin and 0.005–0.02 enzyme units. The incubation was carried out for 30 min at 37°C, at the end of which the mixture was heated for 2 min at 100°C, and then chilled. To this solution 0.05 units of alkaline phosphatase in 0.01 M Tris–HCl, pH 7.5 were added and the mixture was incubated for 30 min at 80°C, after which it was chilled and applied to DEAE-cellulose paper discs (Whatman DE-81). The discs were washed thrice with 5 ml of 0.15 M NH_4HCO_3, then with water, dried and counted in 3 ml toluene-based scintillation fluid.

E. coli alkaline phosphatase was purchased from Worthington (BAPF grade) and preheated to remove RNase [6]. Polynucleotide phosphorylase was prepared as previously described[7].

3. Results

The effect of oligoadenylate chain length on the rate of phosphodiester formation by RNA ligase was examined. The results (table 1) indicate that $(pA)_8$ is the shortest oligoadenylate that still can react while $(pA)_6$ and $(pA)_7$ do not yield any detectable products. The rate of the reaction is markedly increased when the chain length of the oligoadenylates was increased from 8 to 10 and remained constant up to an averarage chain length of 16. However, with longer oligoadenylates there was a decrease in the rate of reaction. The enzymatic joining of $(pA)_9$ and of higher oligoadenylates could be driven to completion (fig. 2). On the other hand, phosphodiester formation with $(pA)_8$ amounted to 15%. The low yield observed

Table 1
The effect of chain length on the rate of circularization of oligo(pA) by RNA ligase

Substrate	Alkaline phosphatase resistant ^{32}P, pmole/30 min
$(pA)_6$	0.0
$(pA)_7$	0.0
$(pA)_8$	0.4
$(pA)_9$	4.0
$(pA)_{10}$	17.2
$(pA)_{11}$	16.9
$(pA)_{16}$	15.0
$(pA)_{30}$	9.2
$(pA)_{100}$	5.4

with this slowly reacting oligonucleotide is probably due to its degradation by contaminating nucleases or phosphatase during the prolonged incubation period required for this reaction.

The kinetic constants were measured for oligoadenylates of varying chain length. A typical Lineweaver–Burk plot for the circularization of $(pA)_{10}$ is shown in fig. 3. The apparent K_m values with oligo(A) of chain length 10 15 30 and 100 were all about 10^{-6}M. The apparent V_{max} values decreased with increasing chain length from 100 to

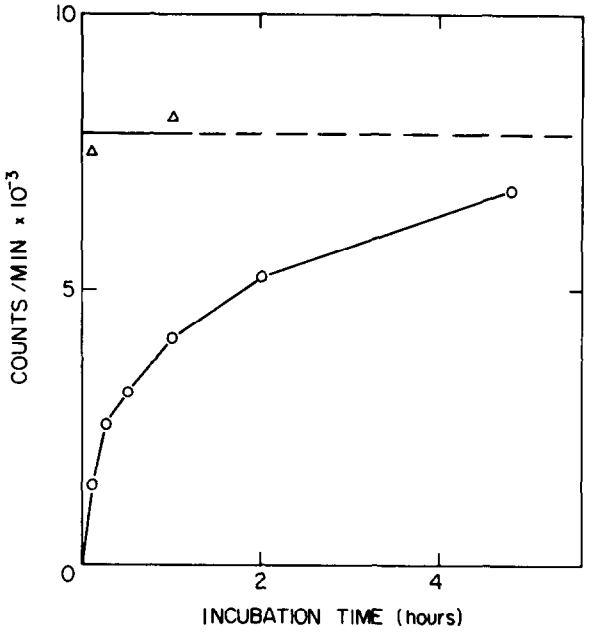

Fig. 2. Time course of RNA ligase reaction with $(pA)_9$. The reaction mixture (0.1 ml) was as described in Materials and methods and contained 2×10^{-6}M 5′-^{32}P-$(pA)_9$ and 16μg RNA ligase. Incubation was at 37°C. Aliquots of 10 μl were withdrawn, as indicated, treated with alkaline phosphatase and adsorbed onto DEAE-cellulose discs as described in methods. (○——○) Aliquots treated with alkaline phosphatase, (△—△) untreated aliquots.

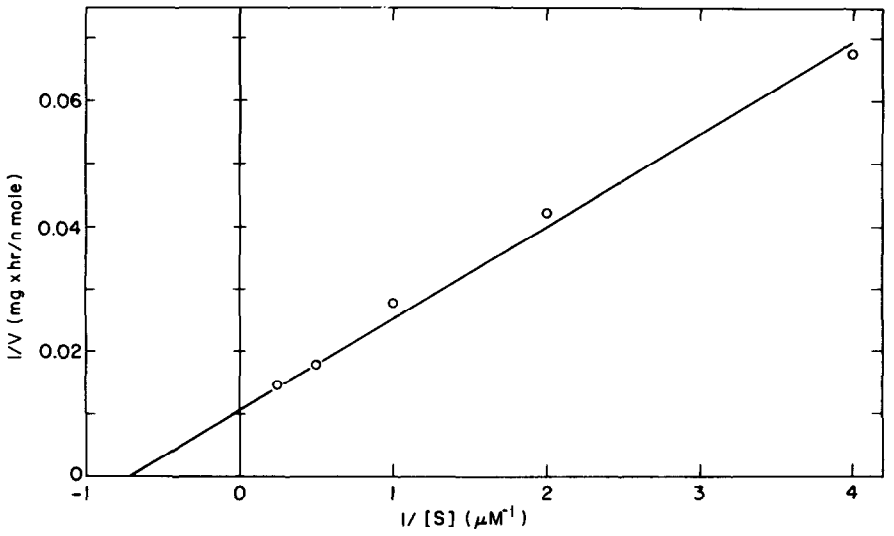

Fig. 3. Effect of $(pA)_{10}$ concentration on the rate of circularization.

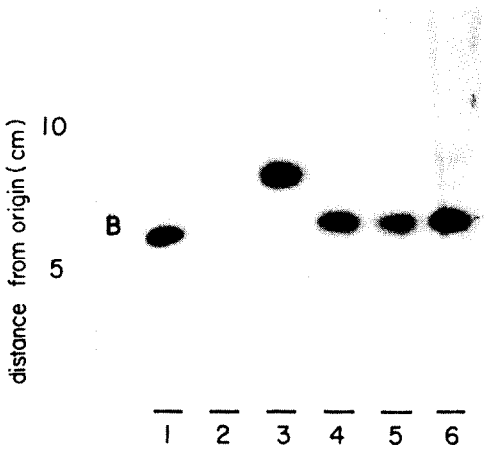

Fig. 4. Analysis of the product of RNA ligase reaction with $(pA)_{10}$. The reaction mixture (0.05 ml) contained 5×10^{-7} M $(pA)_{10}$ and 8 μg RNA ligase. After 2 hr at 37°C the reaction mixture was heated 2 min at 100°C. Aliquots of 15 μl were treated with alkaline phosphatase as described in Materials and methods, or with 35 μl of polynucleotide phosphorylase (1 unit/ml) in 0.01 M potassium phosphate buffer, pH 8.0 for 1 hr at 37°C. The samples were analyzed by electrophoresis on 16% polyacrylamide–7 M urea gel. (1) Reaction mixture minus RNA ligase, not treated. (2) Reaction mixture minus RNA ligase, treated with alkaline phosphatase. (3) Reaction mixture minus RNA ligase, treated with polynucleotide phosphorylase. (4) Complete reaction mixture, not treated. (5) Complete reaction mixture, treated with alkaline phosphatase. (6) Complete reaction mixture, treated with polynucleotide phosphorylase.

30 nmole/mg/hr. With $(pA)_9$ the K_m increased by order of magnitude to 10^{-5} and the V_{max} a value was 15 nmole/mg/hr.

It was important to test whether RNA ligase catalyzed intramolecular circularization of short oligoadenylates, as is the case with long polynucleotides. Fig. 4 demonstrates that the enzyme converts $(pA)_{10}$ to a homogenous product with a slightly faster electrophoretic mobility ($R_{A10} = 1.07$), indicating that there was no doubling of the molecular weight. The slightly enhanced mobility of the product, despite the loss of one negative charge, can be explained by the more compact structure arising from a circular structure. Further proof for the circularity of the product was obtained by enzymatic analysis. Circular $(pA)_{10}$ proved resistant to hydrolysis with alkaline phosphatase hydrolysis, while the substrate of the RNA ligase reaction, $(pA)_{10}$, was totally dephosphorylated. The product was not affected by polynucleotide phosphorylase, while $(pA)_{10}$ was phosphorolyzed into $(pA)_2$. The latter result also shows that the conversion of $(pA)_{10}$ into a cicular form was nearly quantitative.

4. Discussion

In this report we show that RNA ligase catalyzes the circularization of oligoadenylates with a wide range of chain length. It appears that $(pA)_8$ is the shortest substrate that can be circularized by the enzyme. The absence of reaction with $(pA)_6$ and $(pA)_7$ as well as the sluggish rate observed with $(pA)_8$ and $(pA)_9$ may due to inadequate saturation of the enzyme sites or/and the strain induced upon ring closure of short oligonucleotides. This constraint seems to be relieved at a chain length of 10, since there is no further increase in the reaction rate or decrease in the apparent K_m values with higher oligoadenylates. The constant K_m values (10^{-6} M) found over a wide range of chain length, tends to indicate that the enzyme sites recognize only a few nucleotide residues on the chain. The decreasing reaction rates observed with the polyadenylates of high molecular weight (our results and ref. [1]) may reflect the decreasing probability of juxtaposition of the termini with increasing chain length. If this assumption is correct, it may be possible to use the kinetic constants to evaluate the deviation of various polynucleotides from a random coil configuration.

It should be noted that in addition to the intramolecular joining reaction, RNA ligase is able to catalyze the covalent joining of two different ribopolynucleotide chains that are partially hydrogen bonded to each other. Thus, recent experiments have demonstrated the specific joining of two yeast $tRNA^{Phe}$ fragments, containing residues 1–36 and 38–74 respectively [8].

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