Equimolar addition of oligoribonucleotides with T4 RNA ligase

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ABSTRACT

T4 induced RNA ligase will join equimolar concentrations of two oligoribonucleotides, (Ap)₅C and p(Up)₅, to form a single product, (Ap)₅Cp(Up)₅, in high yield. The presence of the 3' phosphate on p(Up)₅ prevents the oligomer from adding to itself. The pH optimum of the reaction is about 7.5, but less of the undesirable adenylated intermediate, App(Up)₅, forms at pH 8.2. The reaction rate is a linear function of oligomer concentration from 3 μM to 0.6 mM. The data suggest that T4 RNA ligase will be a useful enzyme for the synthesis of oligomers of defined sequence.

INTRODUCTION

T4 induced RNA ligase will catalyse the formation of a 3'-5' internucleotide link between a "donor" oligoribonucleotide with a 5' phosphate and another "acceptor" oligoribonucleotide with a 3' hydroxyl (1-3). Since this reaction will occur between a variety of oligomer sequences and chain-lengths at high yields, it suggests that RNA ligase should be a powerful tool for the synthesis of oligoribonucleotides of defined sequence. In this paper the intermolecular reaction between a single pair of oligomers is examined carefully with the intent of finding conditions for the optimal synthesis of complex oligomers with T4 RNA ligase.

It is important to choose the correct type of oligomers to be used in the RNA ligase joining reaction. In previous work (1,3) the ratio of acceptor to donor concentrations was made extremely high (1000 to 1) to prevent intermolecular joining of two donor molecules. Since most of the acceptor molecules in the reaction mixture were not used, the efficiency of the synthetic scheme was limited. Furthermore, differences in ability of oligomers to bind the enzyme can offset the excess of acceptor and still lead to unwanted addition of two donor molecules (3). In this work we will show that by using a donor with a 3' phosphate as well as a 5' phosphate, the enzyme cannot join two donor molecules and the reaction will yield a single intermolecular product. Using 3' blocked donors permits equimolar
addition of two oligomers and thus allows efficient use of both donor and acceptor oligonucleotides.

The model synthetic reaction studied is the equimolar addition of p(Up)₅ to (Ap)₃C to form (Ap)₃Cp(Up)₅. These oligomers are of the general size class which would be useful to join together. Both (Ap)₃C and p(Up)₅ are readily obtained and can easily be separated from one another and from the product by paper chromatography.

**MATERIALS AND METHODS**

T₄ induced RNA ligase was purified from *E. coli* infected with T₄ am 4314, a mutant in the DNA polymerase gene, by the procedure described in Walker et al. (1) except that Sephadex G100 was used instead of G75. A subsequent gradient elution off hydroxyapatite further purified the enzyme (4). The details of this purification will be described elsewhere (5). A unit of RNA ligase activity is defined as 1 nmole of (pA)₁₂ cyclized in 30 min. at 37°C under the following reaction conditions: 1 uM (pA)₁₂, 0.1 mM ATP, 50 mM HEPES pH 7.5, 10 mM MgCl₂ and 0.25 mM dithiothreitol. The cyclization assay procedure has been described previously (1). The RNA ligase preparation used in this work was free of ribonucleases and phosphatases and had a specific activity of 380 units/mg.

Spleen phosphodiesterase (code: SPH), venom phosphodiesterase (code: VPH) and yeast inorganic pyrophosphatase (code: 1PFF) were purchased from Worthington Biochemical Co. In each case the number of enzyme units supplied was assumed to be correct without further assay. Spleen phosphodiesterase was stored at 15 units/ml in 1 mM EDTA and 0.1 M K₂PO₄ pH 6.5. Identification reactions (30 μl) were carried out in the same buffer with 10-50 nmoles of oligonucleotide and 0.1 units of enzyme for 30 min. at 37°C. Venom phosphodiesterase was stored at 10 units/ml in 10 mM MgCl₂ and 20 mM tris-HCl pH 8.5. Identification reactions (30 μl) were carried out in the same buffer with 10-50 nmoles of oligonucleotide and 0.05 units of enzyme for 30 min. at 37°C. Inorganic pyrophosphatase was dialyzed into 50 mM NaCl, 10 mM MgCl₂ and 10 mM tris-HCl pH 7.2 and stored at 200 units/ml. Ribonuclease A and bacterial alkaline phosphatase were purchased from Calbiochem. In both cases identification reactions (30 μl) were carried out in 0.4 M NaCl, 10 mM MgCl₂ and 10 mM tris-HCl pH 8.2 with 10-50 nmoles of oligomer and 2 μg of enzyme for 30 min. at 37°C. T₄ polynucleotide kinase was purified from the same preparation of cells as the T₄ RNA ligase. Details of this purification will be published elsewhere (6). ³H ATP was purchased from New England Nuclear.
(Ap)_3C was obtained by ribonuclease A hydrolysis of poly (A,C), separation of the (Ap)_n Cp series by column chromatography, and treatment of (Ap)_3Cp with alkaline phosphatase (1). Tritium labelled (Ap)_3C (12 Ci/mmole) was obtained by adding a single tritiated cytidine residue to an excess of (Ap)_2A primer with primer dependent polynucleotide phosphorylase (7). This material was mixed with unlabelled (Ap)_3C to obtain the desired specific activity. (Up)_5 was obtained by hydrolyzing poly U (1 mmole) with 1 N KOH for 8 min. at 25°C, neutralizing with 1 N HClO₄ and 10 mM tris-HCl, removing the insoluble KClO₄ by centrifugation and separating the (Up)_n oligomers on a DEAE Sephadex A-25 column (200 ml) with a linear gradient (3,000 ml) of 0.05 to 1.0 M triethyl ammonium bicarbonate. The (Up)_5 peak (80 umoles) was desalted by repeated evaporation in the presence of methanol. p(Up)_5 was synthesized by incubating 0.7 mM (Up)_5, 5 mM ATP, 50 mM tris pH 9.5, 10 mM MgCl₂, 10 mM β mercaptoethanol, 50 µg/ml serum albumin with 7.5 units/ml T4 polynucleotide kinase (8) for one hour at 37°C. The reaction mixture was streaked on Whatman 3 MM paper and the paper was subjected to descending chromatography in 1 M ammonium acetate: 95% ethanol (4/6; v/v). The product (yield = 85%) was eluted from the chromatogram with water after washing the paper with absolute ethanol to remove residual salt. A similar small scale reaction with 0.8 mM (Up)_5, 0.5 mM γ-³²P-ATP (4 Ci/mmole), and 18 units/ml T4 polynucleotide kinase incubated for one hour was used to obtain a supply of ³²P labelled donor which could be mixed with the unlabelled donor to obtain the desired specific activity. The polynucleotide kinase reactions were carried out at a high pH and limiting enzyme concentrations in order to minimize the 3' phosphatase activity present in kinase preparations (6).

Intermolecular RNA ligase reactions between (Ap)_3C and p(Up)_5 were usually carried out in 200 µl polyethylene microcentrifuge tubes (Bel Art). Reaction volume was 30 µl unless stated otherwise. For kinetic studies, 3 µl aliquots were removed at appropriate times and spotted directly on the origin of a Whatman 3 MM paper chromatograph. For fixed time assays, less radioactivity was included in the reaction mixture and the entire 30 µl was spotted on the paper. The paper chromatograms were developed overnight in a descending mode with solvent 1. Solvent 1 was a 3 to 7 mixture (v/v) of 95% ethanol and 1 M ammonium acetate saturated with boric acid at pH 7.0 (9). After developing and drying, nonradioactive oligomers were located by observing the papers in ultraviolet light and radioactivity was located by cutting the paper into one centimeter squares and counting.
them in 4% PPO in toluene in a liquid scintillation counter. In some cases, only squares corresponding to the expected radioactive peaks were counted.

Two other solvents were used with Whatman 3 MM paper in product identification. Solvent 2 was a 6 to 4 mixture (v/v) of 95% ethanol and 1 M ammonium acetate saturated with boric acid at pH 7.0. Solvent 3 was a 6 to 4 mixture (v/v) of ethanol and 1 M ammonium acetate.

RESULTS AND DISCUSSION

An intermolecular reaction between (Ap)$_3$C and p(Up)$_5$ is analyzed by descending chromatography in Fig. 1. The reaction conditions are 50 mM HEPES pH 8.3, 20 mM MgCl$_2$, 3.3 mM dithiothreitol, 10 µg/ml serum albumin, 0.5 mM ATP, 0.1 mM (Ap)$_3$C (specific activity = 330 Ci/mole) 0.1 mM p(Up)$_5$ (specific activity = 100 Ci/mole) and 45 units/ml RNA ligase. The $^{32}$P label present on the 5' terminus of the donor molecule, p(Up)$_5$ (peak 1 in panel A), is converted to two new oligonucleotides (peaks 2 and 4 in panel B) after one hour incubation at 37°C. At the same time, the $^3$H label

Fig. 1. RNA ligase equimolar intermolecular reaction analyzed by paper chromatography in solvent 1 (A) before incubation (B) after 60 minutes at 37°C. Solid line is $^3$H label and dotted line is $^{32}$P label. The solvent front is 38 cm.
present on the 3' terminal cytidine of the acceptor molecule, (Ap)₃C (peak 3 in panel A), is converted to a new oligonucleotide (peak 4 in panel B). Peak 4 can be identified as the intermolecular ligation product of the reaction, (Ap)₃Cp(Up)₅ by a variety of criteria. The ³H label from the acceptor and the ³²P label from the donor comigrate precisely in peak 4. Since 57 percent of the ³H label and 48 percent of the ³²P label is present in peak 4, essentially equal amounts of donor and acceptor make up the peak 4 oligomer. If peak 4 is eluted from the chromatogram in Fig. 1, treated with alkaline phosphatase and rechromatographed, its position is not significantly altered, indicating that the ³²P label is no longer at a terminal position.

In Fig. 2A it is shown that if the material in peak 4 is treated with spleen phosphodiesterase, both the ³H and ³²P labels are converted to 3' CMP. This experiment establishes the internucleotide linkage between the 5' end of the donor molecule and the 3' side of the cytidine on the acceptor molecule. The sensitivity of peak 4 to spleen phosphodiesterase also shows that the 5' end of the oligomer must have a free hydroxyl. Eluted peak 4 is resistant to venom phosphodiesterase, indicating that the 3' end of the product molecule is blocked with a phosphate as expected. However, if peak 4 is first treated with alkaline phosphatase, repurified and then treated with venom phosphodiesterase, the ³²P label is converted to 5' UMP and the ³H label is converted to 5' CMP (Fig. 2B). This confirms that in the peak 4 oligomer the labelled phosphate is still on the 5' side of a uridine residue as it was in the donor molecule. Finally, if peak 4 is treated with ribonuclease A, both the ³²P and ³H label are converted to a compound which comigrates with (Ap)₃Cp, the expected labelled product (Fig. 2C). If alkaline phosphatase is included in this reaction mixture, the 3' terminal labelled phosphate is removed and the ³²P label is found to migrate as inorganic phosphate and the ³H label as (Ap)₃C (Fig. 2D).

Peak 2, the minor product in the intermolecular reaction, can be identified as the adenylated oligonucleotide pAp(Up)₅ on the basis of a variety of experiments. Alkaline phosphatase does not significantly alter the position of peak 2 on the chromatogram indicating that the labelled phosphate is no longer terminal. Reacting peak 2 with venom phosphodiesterase removes the AMP residue and the ³²P migrates in the peak 1 position (data not shown). Peak 2 is resistant to spleen phosphodiesterase as would be expected from the absence of a free 5' hydroxyl in the proposed structure. Presumably the peak 2 oligomer is an intermediate in the RNA
Fig. 2. Enzymatic degradation of peak 4 with (A) spleen phosphodiesterase (B) alkaline phosphatase and then venom phosphodiesterase (C) ribonuclease A (D) ribonuclease A with alkaline phosphatase. Solid lines are $^{3}H$ label and dotted lines are $^{32}P$ label. Panels (A) and (B) use solvent 2. Panels (C) and (D) use solvent 3. Peak 4 remains at the origin in both solvents.

ligase reaction. In analogy to the DNA ligase reaction (10) an enzyme-AMP complex is formed (11), the AMP is transferred to the 5' end of the donor and subsequently displaced by the 3' hydroxyl of the acceptor. Direct demonstration of ATP as the source of the AMP residue is shown in Fig. 3. In this experiment, conditions were identical to those used in
Fig. 3. (A) RNA ligase equimolar intermolecular reaction analyzed by paper chromatography in solvent 3 after 60 minutes of reaction and alkaline phosphatase treatment. (B) Venom phosphodiesterase treatment of intermediate peak in A and analysis in solvent 2. Solid line is $^3$H label and dotted line is $^{32}$P label.

Fig. 1 except that $^3$H ATP (Specific activity = 350 Ci/mole) and nonradioactive (Ap)$_3$C were used. After one hour of incubation, 10 μg of alkaline phosphatase was added and the reaction was incubated at 37° for an additional 30 minutes. The reaction was spotted on paper and chromatographed in solvent 2. In Fig. 3A, the product peak (peak 4 in Fig. 1) migrates close to the origin and contains $^{32}$P label only, the intermediate peak (peak 2 in Fig. 1) migrates close to p(U)p$_5$ and, as expected, contains both $^3$H and $^{32}$P label. The $^{32}$P label in the unreacted donor (peak 1 in Fig. 1) is converted to inorganic phosphate and the unreacted ATP to adenosine by the alkaline phosphatase. The molar ratio of AMP to p(U)p$_5$ in the interme-
diate peak in Fig. 3A is calculated from the $^3$H and $^{32}$P label to be 0.85. Digestion of the intermediate in Fig. 3A with venom phosphodiesterase gave $^3$H 5' AMP and $^{32}$P 5' UMP (Fig. 3B). These data establish the structure of the adenylated oligomer formed by RNA ligase, but do not prove that it is an intermediate in the reaction. Although the amount of $^{32}$P (Up)$_5$ builds up rapidly as would be expected for an intermediate, a considerable amount remains even after long incubation times. Preliminary experiments by Ohtsuka et al. (3) indicate that an adenylated oligomer formed in a similar way can be joined to an acceptor molecule by RNA ligase in the absence of ATP, strongly suggesting an intermediate role for peak 2.

The effects of various alterations in the reaction components upon the formation of product are shown in Table 1. The reaction conditions are identical to those used in Fig. 1, except that only 11 units/ml RNA ligase was used. Under these conditions the amount of product formed is proportional to the incubation time for at least twenty minutes. If the acceptor molecule, (Ap)$_3$C, is omitted from the reaction mixture, or if (Ap)$_3$Cp is substituted, analysis of the paper chromatogram reveals that all the $^{32}$P label remains as (Up)$_5$ in both reactions. The 3' phosphate on the donor molecule prevents self addition when the acceptor is absent and the 3' phosphate on the (Ap)$_3$Cp prevents it from acting as an acceptor. Thus the 3' terminal phosphate group effectively blocks RNA ligase. It is interesting to note that in both of the above reactions, no adenylated donor (peak 2) is formed either. Presumably this implies that the presence of a reactable acceptor is necessary for the adenylation of the donor molecule in the intermediate step in the reaction (12).

TABLE 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Ligase Activity (nmoles product in 15 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Complete System</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Omit (Ap)$_3$C</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td></td>
<td>Omit (Ap)$_3$C, Add (Ap)$_3$Cp</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td></td>
<td>Omit ATP</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>B</td>
<td>No DTT added</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.3 mM DTT</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>3.3 mM DTT</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>33 mM DTT</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>3 mM 8 mercaptoethanol</td>
<td>0.65</td>
</tr>
<tr>
<td>C</td>
<td>100 μM ATP</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>200 μM ATP</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>500 μM ATP</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>1500 μM ATP</td>
<td>0.66</td>
</tr>
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</table>
Not surprisingly, the omission of ATP from the reaction mixture prevents product formation. Under the reaction conditions used here, only a slight excess of ATP is needed to obtain an optimal rate and extent of sealing. When the oligomer concentrations are 100 μM, no effect on the rate is seen when the ATP concentration is increased above 200 μM. The sulfhydryl requirement of RNA ligase has been documented (10) and can also be seen in intermolecular reactions. When no DTT is added, a reduced amount of product is seen, the residual activity perhaps due to the small amount of DTT present in the enzyme solution. The DTT concentration suggested for synthetic reactions (3.3 mM) is about three times higher than is needed for an optimal rate, but concentrations as high as 33 mM may be used without affecting the reaction.

The pH dependence of the RNA ligase reaction is examined in a series of fixed time assays in Fig. 4A. By using a variety of buffers, the pH range from 6.0 to 9.0 could be explored effectively. Each reaction was run at the conditions used in Fig. 1, except that only 11 units/ml RNA ligase was used. In each case, 50 mM buffer was used and the reaction was incubated at 37°C for 15 minutes. Although some differences in yield among the different buffers at a given pH were found, a well defined pH optimum of about 7.9 for the formation of intermolecular product could be seen.

![Fig. 4. Percentage of 32P label from donor incorporated into product (solid marks, solid lines) and intermediate (open marks, broken lines) as a function of pH without (A) and with (B) pyrophosphatase. The buffers were Imidazole (circles), HEPES (triangles) and Glycylglycine (squares).]
Somewhat surprisingly, however, the pH optimum for the formation of adeny-lated oligonucleotide in the same reaction is about 7.5 and is considerably broader. Thus, the relative ratio of product to intermediate is a strong function of pH, being lowest at pH 6.0 and highest at pH 8.3. This general conclusion seems to be correct even if higher enzyme concentrations and longer times are used to drive the reaction to maximal levels. Presumably at lower pH the adenylated intermediate binds less well to the enzyme and does not react with the acceptor molecule. The adenylated oligomer may not be able to rebind the enzyme effectively due to the large excess of ATP present which can adenylate the enzyme. Further experiments investigating the role of the adenylated oligomer in the ligase reaction are in progress. In any case, for the purpose of synthesis, the optimal pH would appear to be about 8.3 where only a small amount of intermediate is made and the specific activity of the enzyme is still relatively high.

In an attempt to increase the rate and extent of the RNA ligase reac-tion, a large excess of inorganic pyrophosphatase was included in the reaction mixture. By destroying pyrophosphate as it was produced during the reaction, the equilibrium of the enzyme adenylation step would be altered and perhaps increase the rate of formation or yield of product. Fixed time assays were carried out exactly as in Fig. 4A, except that 2 units/ml of pyrophosphatase were included in the reaction. This amount of pyrophosphatase was enough to destroy all the pyrophosphate that could be produced in the reaction in a few seconds at all the pH values tested. As is seen by comparing Fig. 4A with Fig. 4B, a relatively large increase in the amount of product formed in 15 minutes is seen between pH 6 and 8.2, but little effect of pyrophosphatase on the yield of product is seen at higher pH values. The pH optimum is therefore shifted to a slightly lower value of 7.5. The stimulation due to pyrophosphatase varies from about two fold at pH 7.8 to nearly ten fold at pH 6.5. Unfortunately for syn-thetic purposes, pyrophosphatase had an even larger effect on the amount of adenylated oligomer formed at low pH values. Thus, at most pH values, the ratio of product to intermediate actually decreased when pyrophosphatase was added. Presumably the pyrophosphatase increased the rate of formation of adenylated enzyme thereby preventing rebinding of adenylated oligomer which had dissociated from the enzyme at low pH. Thus, for optimal syn-thesis conditions at pH 8.3, the addition of pyrophosphatase is generally not useful.
The magnesium ion concentration has a strong effect on the amount of both the product and the adenylated intermediate. Fixed time assays were run as for Fig. 4A at varying concentrations of MgCl₂. The buffer used was 50 mM HEPES pH 7.6 in order to increase the amount of intermediate for more accurate determination. As shown in Fig. 5, at low concentrations of MgCl₂, very little product and a comparatively large amount of adenylated oligomer is formed. As the MgCl₂ concentration is increased, the ratio of product to intermediate increases rapidly. Thus, for optimal conditions for intermolecular synthesis, a high concentration of MgCl₂ should be used.

With optimal reaction conditions for the intermolecular RNA ligase reaction established, it is important to examine the effect of enzyme concentration, time and oligomer substrate concentration on the initial rate and amount of intermolecular product formed. In Fig. 6, the rate of formation of product is measured at several enzyme concentrations. The reaction conditions are identical to those used for Fig. 1. As expected, when it can be accurately measured, the initial rate increases proportionally to the enzyme concentration. However, the final level of product obtained at long incubation times is proportional to the enzyme concentration as well. At low enzyme concentrations, the amount of product reaches an endpoint at about 60 minutes and does not alter after that. The simplest explanation of this is that the enzyme is inactivating during the course of the reaction. If additional enzyme is added at 60 minutes, more product is formed, whereas if additional substrate is added at 60 minutes.
Fig. 6. Kinetics of RNA ligase reaction at varying enzyme concentrations. Insert: initial rate versus enzyme concentration. No additional reaction occurs (experiments not shown). The highest yields of product are obtained if higher enzyme concentrations are added at the beginning of the reaction. Attempts to reduce the apparent enzyme inactivation by adding protein stabilizers have been thus far unsuccessful.

The initial rate of equimolar reaction was measured over a range of oligomer concentrations from 3.3 μM to 600 μM. The ATP concentration was kept at a 3 fold molar excess at the four higher oligomer concentrations and at 100 μM for the two lowest oligomer concentrations (3.3 μM and 10 μM). Other reaction conditions were identical to Fig. 1. At each oligomer concentration kinetics were measured at three enzyme concentrations. Throughout this entire range the initial rate was proportional to the substrate concentration within the accuracy of the measurement (Fig. 7). The amount of adenylated intermediate remained very low at all substrate concentrations and times. The inability to saturate the enzyme in the concentration range tested means that kinetic parameters for the intermolecular reaction could not be obtained from these data. It is clear that for the most efficient use of enzyme in oligomer synthesis, very high concentrations of oligomer should be used. However, useful synthesis may also be carried
Fig. 7. Initial rate of RNA ligase reaction as a function of equimolar oligonucleotide concentration.

out at extremely low oligomer concentrations if enough enzyme is present in the reaction.

The careful study of a single intermolecular RNA ligase reaction provides information for using this enzyme as a general synthetic tool. However, the suggested conditions for the optimal synthesis of \((\text{Ap})_3\text{Cp(Up)}_5\) may not be the same as for the synthesis of other oligomers. We and others (2,3) have noticed rather large effects of the length and composition of the oligomer on the initial rate of reaction. The presence of a 3' phosphate group on the donor molecule will allow a careful measurement of the differences in rate without the interference of the self addition of the donor. We have found that in general good synthetic yields may be achieved by increasing the enzyme and substrate concentrations without altering the buffer. Thus T4 induced RNA ligase is expected to be a powerful tool for the synthesis of oligoribonucleotides of defined sequence.

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