A Stereochemical Study of the Mechanism of Activation of Donor Oligonucleotides by RNA Ligase from Bacteriophage T4 Infected *Escherichia coli*†

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ABSTRACT: RNA ligase from bacteriophage T4 infected *Escherichia coli* catalyzes the activation of adenosine 3′,5′-bisphosphate (representing the donor oligonucleotide) by adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate with retention of configuration at P_α. Since single-step enzyme-catalyzed nucleotidyl transfer reactions proceed with inversion, this stereochemical result provides support for a double displacement mechanism involving an adenylyl-enzyme intermediate as proposed previously from isotope exchange experiments.

T4 RNA ligase, which is found in *Escherichia coli* after infection with T-even bacteriophage, was originally detected during a study of T4 DNA ligase by its ability to cyclize homopolyribonucleotides with a 3′-terminal hydroxyl group and a 5′-terminal phosphate. The formation of the 3′,5′-phosphodiester bond is dependent on ATP, which is concomitantly converted into AMP and inorganic pyrophosphate (PP_i) (Leis et al., 1972; Silber et al., 1972). The enzyme also catalyzes the ATP-dependent formation of a 3′,5′-phosphodiester bond between an oligoribonucleotide with a 3′-hydroxyl group (the acceptor) and the 5′-terminal phosphate of a second oligoribonucleotide (the donor). Intermolecular ligation is observed if the donor is too short to cyclize (Kaufmann et al., 1974) or if the 3′-hydroxyl group of the donor is protected, for example, as a phosphate ester (Uhlenbeck & Cameron, 1977). The acceptor may be as small as a trinucleotide diphosphate and the donor as small as a nucleoside 3′,5′-bisphosphate (Kaufmann & Kallenbach, 1975; England & Uhlenbeck, 1978; Kikuchi et al., 1978). The enzyme is highly specific for ATP, no other ribonucleoside triphosphate being capable of supporting the reaction (Cranston et al., 1974).

In the absence of oligonucleotide substrates the enzyme catalyzes ATP–[^{32}P]PP_i exchange and an adenylated enzyme intermediate can be isolated (Cranston et al., 1974), in which 1 mol of AMP is bound to the enzyme through the ϵ-amino group of a lysine residue (Juodka et al., 1980). In the presence of a donor oligoribonucleotide and ATP the enzyme catalyzes the formation of A-5′pp5′-X, where X is a mono- or oligoribonucleotide. Under favorable conditions the adenylated donor can be isolated (Ohtsuka et al., 1976; Sninsky et al., 1976). It has been shown that chemically synthesized adenylated donors, A-5′pp5′-X, are chemically competent intermediates, i.e., T4 RNA ligase will catalyze the formation of a 3′,5′-phosphodiester bond in the presence of an acceptor (England et al., 1977). The reaction has been formulated therefore in terms of a three-step mechanism (Uhlenbeck & Gumport, 1982), viz.

$$E + MgATP \rightleftarrows E\text{-}pA + MgPP_i$$
$$E\text{-}pA + pX \rightleftarrows E{\cdot}A\text{-}5'ppX$$
$$E{\cdot}A\text{-}5'ppX + Y \rightleftarrows E + AMP + YpX$$

No kinetic analysis of this mechanism has been undertaken, and it is not known whether the adenylated enzyme (E-pA) and the enzyme-bound adenylated donor (E·A-5′ppX) are kinetically competent intermediates. However, since enzyme-catalyzed displacement reactions at phosphate esters have invariably been found to occur with inversion of configuration at phosphorus (Knowles, 1980; Lowe et al., 1981; Lowe, 1983), a stereochemical investigation should allow the three-step mechanism to be scrutinized. In a recent study the stereochemical course of the third step of the reaction mechanism was investigated by using the chemically synthesized dinucleoside thiopyrophosphate App(S)I. Only the S_P diastereoisomer behaved as an activated donor for the oligonucleotide acceptor ApApA. The product ApApAp(S)I was shown to have the R_P configuration at the thiophosphate group; hence the 3′,5′-phosphodiester bond must be formed with inversion of configuration indicative of an "in line" displacement of the adenylated donor (Bryant & Benkovic, 1981, 1982).

The activation of the donor substrate by MgATP is a nucleotidyl transfer reaction, and if the three-step mechanism is correct (involving an E-pA intermediate), the activation of the donor substrate should occur with retention of configuration at P_α of ATP. With one exception the stereochemical course of all nucleotidyl transferases that have been investigated proceeds with inversion of configuration of P_α of the nucleoside 5′-di- or triphosphate. These include enzymes that esterify carboxy groups such as acetyl coenzyme A (acetyl-CoA) synthetase (Midelfort & Sarton-Miller, 1978) and the aminoacyl-tRNA synthetases (Langdon & Lowe, 1979; Lowe et al., 1983a,b; Lowe & Tansley, 1984a,b; Connolly et al., 1984; Harnett et al., 1985), ATP sulfurylase, which activates inorganic sulphate (Bicknell et al., 1982), enzymes that catalyze the formation of phosphodiester bonds such as RNA polymerase (Burgers & Eckstein, 1978; Eckstein, 1976), DNA polymerase (Brody & Frey, 1981; Burgers & Eckstein, 1979a), tRNA nucleotidyl transferase (Eckstein et al., 1977), adenylate cyclase (Coderre & Gerlt, 1980; Gerlt et al., 1980), polynucleotide phosphorylase (Burgers & Eckstein, 1979b), and enzymes that catalyze the formation of pyrophosphate diesters such as UDPglucose pyrophosphorylase (Sheu & Frey, 1978) and NAD^+ pyrophosphorylase (Lowe & Tansley, 1983). The one exception is galactose-1-phosphate uridylyltransferase, which catalyzes uridylyl transfer between galactose 1-phosphate and glucose 1-phosphate (Sheu et al., 1979). It has been suggested that because of the structural and electrostatic similarity of these sugar phosphates, it would be an evolutionary advantage if a suitably located enzymic amino acid provided a refuge for the uridylyl moiety, the new bond maintaining its transfer potential while the sugar phosphates

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interchanged at a common binding site (Sheu et al., 1979). This attractive suggestion could not apply to T4 RNA ligase, however, since PP_i and the oligoribonucleotide donor have grossly dissimilar structures.

In order to demonstrate whether an adenylyl-enzyme intermediate is involved in the activation of a donor substrate, we have undertaken a stereochemical experiment. The expectation is that if an adenylyl-enzyme intermediate is on the reaction pathway, activation should occur with retention of configuration at phosphorus, whereas if direct adenylation of the donor substrate occurs, then inversion of configuration should be observed.

Materials and Methods

RNA ligase from T4-infected *E. coli* was purchased from Pharmacia P-L Biochemicals (Milton Keynes, U.K.) and was stored at −20 °C in a buffer consisting of 50% glycerol, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (0.02 M, pH 7.5), 2-mercaptoethanol (5 mM), ethylenediaminetetraacetic acid (EDTA) (0.1 mM), and ATP (50 μM).

Inorganic pyrophosphatase and snake venom phosphodiesterase were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.) and BDH (Poole, Dorset, U.K.), respectively. Deuterium oxide (99.8 atom % ^2H) was obtained from Fluorochem Ltd. (Glossop, Derbyshire, U.K.). High-grade deionized water, used in the preparation of all buffers, was obtained from a Milli-Q2 water purification system (Millipore Ltd., Harrow, Middlesex, U.K.).

Analysis of nucleotides was performed by ion-exchange chromatography using a fast protein liquid chromatography (FPLC) system (Pharmacia, Hounslow, U.K.) and the polyanion SI resin. Adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate was synthesized as described by Lowe & Tansley (1984b) and had the following isotopic composition (calculated from ^{31}P NMR spectra): 11% ^{16}O, 52% ^{17}O, and 37% ^{18}O at the ^{17}O site and 92% ^{18}O per ^{18}O site. It had in excess of 95% of the *S* enantiomer.

Incubation of Adenosine 3′,5′-Bisphosphate and Adenosine 5′-[(S)-α-^{17}O,α,α-$^{18}O_2$]Triphosphate with RNA Ligase and Inorganic Pyrophosphatase. RNA ligase (T_4-infected *E. coli*) (750 units, 75 μL) and inorganic pyrophosphatase (22 units) were added to a solution of 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (Hepes) (5 mL, 50 mM, pH 7) containing magnesium chloride (20 mM), adenosine 3′,5′-bisphosphate (20 mM), adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]-triphosphate (10 mM, 50 μmol), dithiothreitol (3.3 mM), and bovine serum albumin (0.05 mg). The solution was incubated at 37 °C and the reaction monitored by FPLC. When the reaction was complete (20 h), the reaction solution was diluted (20 mL) and applied to a column of DEAE-Sephadex A-25 (10 mL) that had been equilibrated with triethylammonium bicarbonate buffer (100 mM, pH 7.8), and the column was eluted with a linear gradient of triethylammonium bicarbonate buffer (100–600 mM, pH 7.8, 24 h, 25 mL/h). [^{16}O,^{17}O,^{18}O]Ap_2Ap (31 μmol, 62%) and adenosine 3′,5′-bisphosphate (60 μmol) were eluted together and were freed from buffer by addition and evaporation of methanol (3 × 10 mL): NMR (50% D_2O, p"H" 9) δ_P 0.96 (s, pA*p*), 0.88 (s, Ap_2A*p*), 0.64 (s, *p*Ap), and −14.16 (AB quartet, J_{AB} = 20 Hz, Ap_2Ap).

Incubation of [^{16}O,^{17}O,^{18}O]Ap_2Ap with Snake Venom Phosphodiesterase. Snake venom phosphodiesterase (*Crotalus adamanteus*, 15 mg, 2.25 units) was added to a solution of [^{16}O,^{17}O,^{18}O]Ap_2Ap (31 μmol) and adenosine 3′,5′-bisphosphate (60 μmol) in Tris-HCl buffer (20 mL, 100 mM, pH 8.9), and the solution was incubated at 37 °C. The reaction was monitored by FPLC and was complete after 3 h. The reaction solution was applied to a column of DEAE-Sephadex A-25 (10 mL) that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.8) and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–200 mM, pH 7.8, 24 h, 25 mL/h). [^{16}O,^{17}O,^{18}O]AMP (20 μmol) was freed from buffer by addition and evaporation of methanol (3 × 10 mL) and then cyclized and methylated for analysis by ^{31}P NMR spectroscopy of the chirality at phosphorus as described by Jarvest et al. (1981).

Table I: Observed and Calculated Relative ^{31}P NMR Intensities[a]

labeled triester	equatorial triester			axial triester		
	obsd	calcd		obsd	calcd	
		retention	inversion		retention	inversion
MeO—P═O	0.38	0.37	0.37	0.35	0.34	0.34
Me●—P═O	0.65	0.65	1.00	1.00	1.00	0.59
MeO—P═●	1.00	1.00	0.65	0.59	0.59	1.00
Me●—P═●	0.37	0.35	0.35	0.40	0.35	0.35

[a] Observed relative peak intensities of the ^{31}P NMR resonances (from Figure 1) of the cyclized and methylated 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by activating adenosine 3′,5′-bisphosphate with adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate and RNA ligase and by hydrolyzing [^{17}O,^{18}O]Ap_2Ap with snake venom phosphodiesterase. Comparison is made with the values calculated for retention and inversion of configuration on the basis of the known isotopic composition of adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate. ● = ^{18}O.

^{31}P NMR Spectra. ^{31}P NMR spectra were recorded at 101.256 MHz on a Bruker AM-250 Fourier transform spectrometer with quadrature detection. Signal averaging was performed by an Aspect 2000 computer interfaced with the spectrometer. Field frequency locking was provided by the deuterium resonance of D_2O or CD_3CN. Parameters used for the spectra of the methyl esters of cyclic AMP were as follows: sweep width, 1500 Hz; acquisition time, 2.73 s; pulse width, 10.0 μs; broad-band proton decoupling; Gaussian multiplication (line broadening, −0.45; Gaussian broadening, 0.5) of the free induction decay (FID) in 8K and Fourier transform in 32K.

Cyclization of [^{16}O,^{17}O,^{18}O]AMP proceeds with inversion of configuration at phosphorus (Jarvest et al., 1981). Calculation of the expected ratios of relative peak intensities in the ^{31}P NMR spectrum of the isotopomers of the axial and equatorial methyl esters of cAMP requires, in addition to the values of isotopic composition, the enantiomeric excess of adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate and the loss of label during cyclization. A computer program, written by Dr. A. Davies for a Research Machines 380Z microcomputer, which steps through values for the loss of ^{17}O and ^{18}O label on cyclization and the enantiomeric excess of the *S* enantiomer of adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate and prints out the sum of the squares of the difference between the calculated and observed ratios, was used to give the best fit to the experimental data (Table I).

Results and Discussion

Several routes have recently been reported for the synthesis of nucleoside 5′-diphosphates made chiral at P_α by isotopic substitution (Sammons & Frey, 1982; Connolly et al., 1982; Lowe et al., 1982; Iyengar et al., 1984). For this investigation, however, adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate (Lowe & Tansley, 1984b) was used in order to simplify the stereochemical analysis (see below).

Adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate was incubated with T4 RNA ligase in the presence of adenosine 3′,5′-bisphosphate under conditions that favored the production of [^{17}O,^{18}O]A-5′pp5′-Ap (Uhlenbeck & Cameron, 1977).

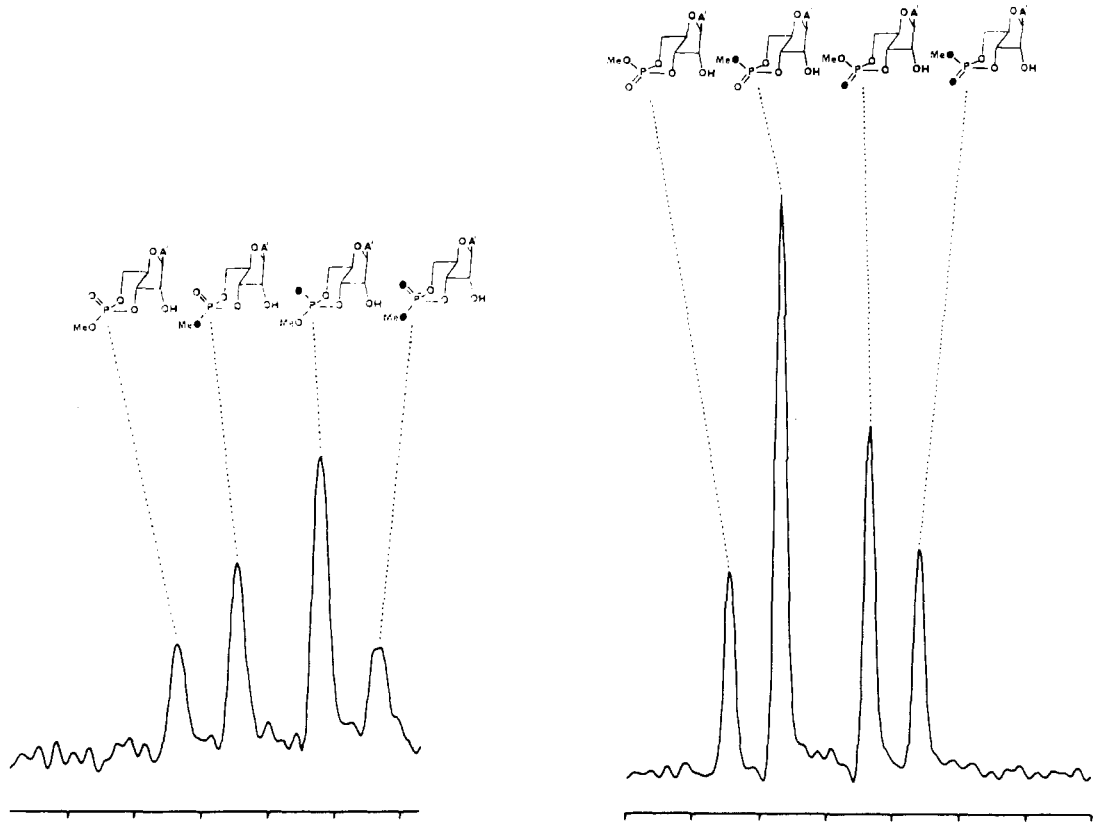
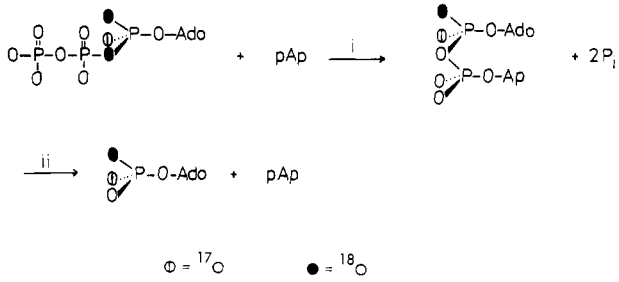


FIGURE 1: ^{31}P NMR spectrum of the equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by activating adenosine 3′,5′-bisphosphate with adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate and hydrolyzing [^{17}O,^{18}O]Ap_2Ap with snake venom phosphodiesterase. The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ triesters to the $^{18}O_{ax}$,$^{16}O_{eq}$ triesters shows that 5′-[^{16}O,^{17}O,^{18}O]AMP has the R_P configuration, and hence the reaction has proceeded with retention of configuration of P_α of ATP as indicated in Scheme I. The axial triesters are on 0.5 of the intensity scale as that for the equatorial triesters. Each division represents δ 0.02. ● = ^{18}O, A′ = *N*-methyladenine.

Scheme I: Stereochemical Course of Activation of Adenosine 3′,5′-Bisphosphate by Adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]Triphosphate Catalyzed by RNA Ligase from T4-Infected *E. coli*[a]



[a] Snake venom phosphodiesterase hydrolyzes phosphodiester bonds with retention of configuration at phosphorus. Reagents: (i) T4 RNA ligase, inorganic pyrophosphatase; (ii) snake venom phosphodiesterase, H_2O.

Inorganic pyrophosphatase was added to the reaction mixture in order to prevent the back reaction (Scheme I). In this way [^{17}O,^{18}O]A-5′pp5′-Ap was obtained in 62% yield (but not separated from excess adenosine 3′,5′-bisphosphate).

In order to determine the chirality of the isotopically labeled phosphate residue, [^{17}O,^{18}O]A-5′pp5′-Ap was hydrolyzed by snake venom phosphodiesterase to give [^{16}O,^{17}O,^{18}O]AMP and adenosine 3′,5′-bisphosphate, which were separated by ion-exchange chromatography. Although snake venom phosphodiesterase may catalyze attack at either of the phosphate residues, since it catalyzes the hydrolysis of phosphodiester bonds with retention of configuration at phosphorus (Jarvest & Lowe, 1981; Mehdi & Gerlt, 1981), [^{16}O,^{17}O,^{18}O]AMP will have the same configuration irrespective of whether the ^{16}O arises from the bridge oxygen or from the solvent water (Scheme I). It was for this reason that we used adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate for this stereochemical analysis.

The chirality at phosphorus of [^{16}O,^{17}O,^{18}O]AMP was determined by our established procedure (Jarvest et al., 1981). The ^{31}P NMR spectrum after cyclization and methylation is shown in Figure 1. From the known isotopic content and enantiomeric excess of the adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate used, it was possible to calculate the expected relative peak intensities of the ^{31}P NMR resonances of the axial and equatorial triesters for the reaction proceeding with retention or inversion of configuration at phosphorus. Comparison of the observed and calculated relative peak intensities (Table I) shows that T4 RNA ligase catalyzes the adenylation of adenosine 3′,5′-bisphosphate stereospecifically (within experimental error) with retention of configuration at P_α. Since single phosphoryl and nucleotidyl transfer reactions have been found to occur with inversion of configuration at phosphorus (Knowles, 1980; Lowe, 1983), overall retention is in accord with a double displacement mechanism. The stereochemical evidence therefore provides powerful support for the pathway outlined in the introduction in which the oligonucleotide donor is adenylated by MgATP via an adenylyl-enzyme intermediate.

Registry No. pAp, 1053-73-2; 5′ATP, 56-65-5; RNA ligase, 37353-39-2.

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