References
Altona, C., & Sundaralingam, M. (1973) *J. Am. Chem. Soc. 95*, 2333–2344.
Arnott, S. (1970) *Prog. Biophys. Mol. Biol. 21*, 267–318.
Baginski, E. S., Foa, P. P., & Zak, B. (1967) *Clin. Chem. (Winston-Salem, N.C.) 13*, 326–332.
Chen, P. S., Jr., Torribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
Cheng, D. M., & Sarma, R. H. (1977) *J. Am. Chem. Soc. 99*, 7333–7348.
Dailey, B. P., & Shoolery, J. N. (1955) *J. Am. Chem. Soc. 77*, 3977–3981.
Davies, D. B., & Danyluk, S. S. (1974) *Biochemistry 13*, 4417–4434.
De Clercq, E., Fukui, T., Kakiuchi, N., Ikehara, M., Hattori, M., & Pfleiderer, W. (1979) *Cancer Lett. (Shannon, Irel.) 7*, 27–37.
De Clercq, E., Stollar, B. D., Hobbs, J., Fukui, T., Kakiuchi, N., & Ikehara, M. (1980) *Eur. J. Biochem. 107*, 279–288.
Drake, A. F., Mason, S. F., & Trim, A. R. (1974) *J. Mol. Biol. 86*, 727–739.
Drew, H. R., Wing, R. M., Takano, T., Broka, C., Tanaka, S., Itakura, K., & Dickerson, R. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2179–2183.
Evans, F. E., & Sarma, R. H. (1974) *J. Biol. Chem. 249*, 4754–4759.
Ezra, F. S., Lee, C.-H., Kondo, N. S., Danyluk, S. S., & Sarma, R. H. (1977) *Biochemistry 16*, 1977–1987.
Fujimoto, M., Kuninaka, A., & Yoshino, H. (1974) *Agric. Biol. Chem. 38*, 777–783.
Fukui, T., Kakiuchi, N., & Ikehara, M. (1982) *Biochim. Biophys. Acta 697*, 174–177.
Giessner-Prettre, C., & Pullman, B. (1976) *Biochem. Biophys. Res. Commun. 70*, 578–581.
Guschlbauer, W., & Jankowski, K. (1980) *Nucleic Acids Res. 8*, 1421–1433.
Ikehara, M., & Miki, H. (1978) *Chem. Pharm. Bull. 26*, 2449–2453.
IUPAC–IUB Commission on Biochemical Nomenclature (1970) *Biochemistry 9*, 4022–4027.
IUPAC–IUB Commission on Biochemical Nomenclature (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2222–2230.
Johnson, N. P., & Schleich, T. (1974) *Biochemistry 13*, 981–987.
Lee, C.-H., Ezra, F. S., Kondo, N. S., Sarma, R. H., & Danyluk, S. S. (1976) *Biochemistry 15*, 3627–3638.
Olsthoorn, C. S. M., Haasnoot, C. A. G., & Altona, C. (1980a) *Eur. J. Biochem. 106*, 85–95.
Olsthoorn, C. S. M., Bostelaar, L. J., van Boom, J. H., & Altona, C. (1980b) *Eur. J. Biochem. 112*, 95–110.
Quigley, G. J., Seeman, N. C., Wang, A. H.-J., Suddath, F. L., & Rich, A. (1975) *Nucleic Acids Res. 2*, 2329–2341.
Seeman, N. C., Rosenberg, J. M., Suddath, F. L., Kim, J. J. P., & Rich, A. (1976) *J. Mol. Biol. 104*, 109–144.
Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., & Kyogoku, Y. (1979) *Tetrahedron Lett.*, 4073–4076.
Uesugi, S., Takatsuka, Y., Ikehara, M., Cheng, D. M., Kan, L. S., & Ts'o, P. O. P. (1981a) *Biochemistry 20*, 3056–3062.
Uesugi, S., Shida, T., & Ikehara, M. (1981b) *Chem. Pharm. Bull. 29*, 3573–3585.
Warshaw, M. M., & Tinoco, I., Jr. (1965) *J. Mol. Biol. 13*, 54–64.
Warshaw, M. M., & Cantor, C. R. (1970) *Biopolymers 9*, 1079–1103.

Phosphorothioate Substrates for T4 RNA Ligase†

F. R. Bryant and S. J. Benkovic*

ABSTRACT: Stereochemical details of the T4 RNA ligase reaction mechanism have been delineated by examining the reactivity of phosphorothioate analogues in the adenosine 5′-triphosphate (ATP) dependent and ATP-independent RNA ligase reactions. Only the S_P isomer of the diastereomeric dinucleoside thiopyrophosphate, App(s)I, was active as an activated donor substrate in the ATP-independent RNA ligase reaction. The thiophosphodiester linkage in the ligation product, ApApAp(s)I, that is formed by the reaction of App(s)I (S_P) with the oligonucleotide acceptor, ApApA, was shown to have the R_P configuration. This indicates that phosphodiester bond formation occurs by a direct, nucleophilic displacement of AMP from App(s)I by the 3′-hydroxyl group of ApApA with inversion of configuration at phosphorus. The adenylylated intermediate, App(s)Ap, that is formed from the phosphorothioate donor, p(s)Ap, in the ATP-dependent RNA ligase reaction was shown to have the same stereochemical configuration as is required for the ATP-independent RNA ligase reaction. These results indicate that RNA ligase maintains a preferred chirality at phosphorus through the adenylylation and ligation steps of the reaction mechanism. An unusual result is the accumulation of adenosine cyclic 2′,3′-phosphate 5′-phosphorothioate in the ATP-dependent RNA ligase reaction employing the donor p(s)Ap when the acceptor ApApA is present. This observation suggests that there are two distinct but reactive modes for donor molecules.

RNA ligase from bacteriophage T4-infected *Escherichia coli* catalyzes the ATP-dependent formation of a 3′–5′ phosphodiester bond between the 3′-hydroxyl group of one oligoribonucleotide (the acceptor) and the 5′-phosphoryl group of a second oligoribonucleotide (the donor), thereby producing a covalently joined product. This reaction occurs via a minimal three-step mechanism outlined in Scheme I.

Scheme I

$$E + ATP \rightleftarrows E\text{–}pA + PP_i \quad (1)$$

$$E\text{–}pA + pN_n \rightleftarrows E[A\text{-}5'pp5'\text{-}N_n] \quad (2)$$

$$E[A\text{-}5'pp5'\text{-}N_n] + M_m \rightleftarrows M_mpN_n + AMP + E \quad (3)$$

$$n \geq 1 \qquad m \geq 3$$

† From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received April 12, 1982.* This investigation was supported by a grant from the National Institutes of Health (GM13306).

0006-2960/82/0421-5877$01.25/0

In the first step, RNA ligase reacts with ATP to form an adenylylated enzyme with release of inorganic pyrophosphate (Cranston et al., 1974). In the following step, the adenylyl group is transferred from the enzyme to the 5′-phosphoryl group of the donor oligoribonucleotide to form an adenylylated molecule having a 5′–5′ phosphoanhydride bond (Ohtsuka et al., 1976; Sninsky et al., 1976). The third step involves reaction of the 3′-hydroxyl group of the acceptor oligoribonucleotide with the activated 5′-phosphoryl group of the donor, resulting in the formation of a phosphodiester linkage between the two oligoribonucleotides with the concomitant release of AMP (Sugino et al., 1977). This last step may proceed mechanistically through a direct nucleophilic displacement involving A-5′pp5′-N_n and the acceptor species or through a covalent E–pN intermediate, a counterpart to E–pA in step 1 (see Scheme I).

RNA ligase will utilize acceptor molecules as small as trinucleoside diphosphates (m = 3) (England & Uhlenbeck, 1978) and donor molecules as small as nucleoside 3′,5′-bisphosphates (n = 1) (Kikuchi et al., 1978). The enzyme shows a strong preference for ATP as the free energy source for phosphodiester bond formation with no other ribonucleoside triphosphates being capable of supporting the reaction (Cranston et al., 1974). In addition to the normal ATP-dependent reaction, RNA ligase will also catalyze an ATP-independent reaction between an acceptor molecule and a variety of dinucleoside pyrophosphates having the general structure A-5′pp5′-X, where X can be any ribonucleoside (England et al., 1977). The dinucleoside pyrophosphates presumably function as analogues of the adenylylated oligoribonucleotide intermediate, thereby allowing the enzyme to bypass the ATP-dependent adenylylation step and proceed directly to the final phosphodiester bond-forming step. Although RNA ligase will tolerate a wide variation in the nonadenosine portion of the dinucleoside pyrophosphate, the specificity for the adenosine portion—the portion eliminated as AMP—is very high, consistent with the high specificity exhibited by RNA ligase for ATP in the ATP-dependent reaction. In the present study, we were able to delineate stereochemical details of the RNA ligase reaction mechanism by studying the fates of the chiral dinucleoside α-thiopyrophosphates, App(s)A[1] and App(s)I in the ATP-independent RNA ligase reaction and the prochiral phosphorothioate donor p(s)Ap in the ATP-dependent reaction. A preliminary account of some of this work has been reported (Bryant & Benkovic, 1981).

Experimental Procedures

Materials. T4 RNA ligase was a gift from Professor Olke Uhlenbeck (University of Illinois) and had a specific activity of 3100 units/mg. One unit of RNA ligase activity is defined as 1 nmol of $(pA)_{12}$ cyclized in 30 min at 37 °C under the following reaction conditions: 1 μM $(pA)_{12}$, 0.1 mM ATP, 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, and 0.25 mM dithiothreitol (Walker et al., 1975). Ap(s)A (R_P and S_P) was furnished by Dr. John Marlier of this group (Marlier & Benkovic, 1980). pAp(s)A (R_P and S_P) was prepared from Ap(s)A (R_P and S_P) with polynucleotide kinase (F. R. Bryant and S. J. Benkovic, unpublished results). Phosphodiesterase I (*Crotalus adamanteus* venom), phosphodiesterase II (bovine spleen), inorganic pyrophosphatase (yeast), alkaline phosphatase (*Escherichia coli*), ribonuclease T_2 (*Aspergillus oryzae*), adenylic acid deaminase (rabbit muscle), pyruvate kinase (rabbit muscle), and lactic dehydrogenase (rabbit muscle) were from Sigma. ATP (disodium salt), NADH (disodium salt), PEP (trisodium salt), and 2′(3′)-AMP (sodium salt) were also from Sigma. ApApA (ammonium salt) was from Boehringer Mannheim. Adenosine, diphenyl phosphorochloridate, and 2,6-dibromobenzoquinone-4-chloroimide were from Aldrich. Thiophosphoryl chloride was from Alfa. DEAE-Sephadex A-25 was from Pharmacia, DEAE-cellulose DE-52 was from Whatman, and Dowex 50X4-400 (200–400 mesh) was from Bio-Rad. PEI–cellulose-F thin-layer plates were from MC/B. Triethylammonium bicarbonate solution (1 M) was prepared by bubbling carbon dioxide through a sintered glass bubbler into a cooled, aqueous solution of triethylamine (1 M) until the pH reached 7.5. Dimethylformamide and pyridine were dried by distillation from calcium hydride; dioxane was purified by a published procedure (Gordon & Ford, 1972). All other organic reagents, buffers, and inorganic salts were commercially available, reagent-grade chemicals. Doubly distilled deionized water was used throughout.

General Procedures. UV spectra were obtained on a Cary Model 118 spectrophotometer. Spectrophotometric enzyme assays and concentration measurements were performed on a Gilford Model 240 spectrophotometer. ^{31}P NMR spectra were obtained on a JEOL PS-100-FT spectrometer at 40.29 MHz, and chemical shifts (δ) are reported in ppm relative to an 85% H_3PO_4 standard, downfield shifts being designated as positive. pH measurements were made on a Radiometer Model 22 pH meter equipped with a Model PHA 630 Pa scale expander at 25 °C. Thin-layer chromatography was carried out on PEI–cellulose thin-layer plates that were pretreated with a fluorescent indicator and developed with 0.75 M potassium phosphate buffer (pH 3.5): UV absorbing compounds were visualized under UV light (254 nm); sulfur-containing compounds were detected with the sulfur-sensitive reagent DQC (2,6-dibromobenzoquinone-4-chloroimide, 1% in acetic acid) (Stenerson, 1971). DEAE-cellulose DE-52 and DEAE-Sephadex A-25 were prepared in the bicarbonate form by first soaking them in 1 M ammonium (or triethylammonium) bicarbonate overnight and then washing them with several volumes of the starting buffer of the elution gradient. DEAE-cellulose and DEAE-Sephadex column elutions were monitored spectrophotometrically at 260 nm.

Syntheses. (*A*) *Adenosine(5′)-α-thiodiphospho(5′)-adenosine* (**3a**). AMPS was prepared according to the procedure of Murray & Atkinson (1968). AMPS (0.5 mmol, disodium salt) was dissolved in water (2 mL) and the solution was passed through a Dowex 50X4 column (1.2 × 10 cm, pyridinium form). The eluant was evaporated to give the pyridinium salt of AMPS. Trioctylamine (0.22 mL, 0.5 mmol) and methanol (10 mL) were added, and the mixture was

[1] Abbreviations: pAp, adenosine 3′,5′-diphosphate; ApApA, adenylyl(3′–5′)adenylyl(3′–5′)adenosine; ApApApA, adenylyl(3′–5′)-adenylyl(3′–5′)adenylyl(3′–5′)adenosine; AMPS, adenosine 5′-phosphorothioate; p(s)Ap, adenosine 3′-phosphate 5′-phosphorothioate; p(s)A>p, adenosine 2′,3′-phosphate 5′-phosphorothioate; Ap(s)A, adenosine(3′)-thiophospho(5′)adenosine; ADPβS, adenosine 5′-(2-thiodiphosphate); App(s)A, adenosine(5′)-α-thiodiphospho(5′)adenosine; IMPS, inosine 5′-phosphorothioate; IDPαS, inosine 5′-(1-thiodiphosphate); ApApAp(s)I, adenylyl(3′–5′)adenylyl(3′–5′)adenosine(3′)-thiophospho(5′)inosine; App(s)I, inosine(5′)-α-thiodiphospho(5′)-adenosine; App(s)Ap, adenosine 3′-phosphate 5′-[α-thiodiphospho(5′)-adenosine]; p(s)AppA, adenosine 5′-phosphorothioate 3′-[diphospho-(5′)adenosine]; ApApAp(s)Ap, adenylyl(3′–5′)adenylyl(3′–5′)-adenosine(3′)thiophospho(5′)adenosine 3′-phosphate; HPLC, high-pressure liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; DEAE, diethylaminoethyl; PEI, poly(ethylenimine); TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; BSA, bovine serum albumin.

stirred until a clear solution was obtained. The solution was evaporated, and the resulting residue was evaporated with anhydrous dimethylformamide (3 × 15 mL). The residue was dissolved in anhydrous dioxane (2 mL); then diphenyl phosphorochloridate (0.15 mL, 0.75 mmol) and tributylamine (0.25 mL, 1.0 mmol) were added, and the mixture was stirred for 3 h at room temperature. The mixture was evaporated, anhydrous diethyl ether (10 mL) and petroleum ether (30 mL) were added, and the mixture was left for 30 min at 0 °C. The ethers were decanted, the remaining material was dissolved in anhydrous dioxane (1 mL), and the solution was evaporated to dryness.

AMP (1.0 mmol, disodium salt) was dissolved in water (4 mL) and the solution was passed through a Dowex 50X4 column (1.2 × 20 cm, pyridinium form). The eluant was evaporated to give the pyridinium salt of AMP. Trioctylamine (0.44 mL, 1.0 mmol) and methanol (20 mL) were added, and the mixture was stirred until a clear solution was obtained. The solution was evaporated, and the resulting residue was evaporated with anhydrous dimethylformamide (3 × 15 mL). The residue was dissolved in pyridine (4 mL), and the solution was added to the dried activated AMPS. After an 8-h stirring at room temperature, the solution was evaporated, and the residue was stirred with diethyl ether (10 mL). The ether was decanted, and the remaining material was dissolved in water (50 mL).

The aqueous solution was applied to a DEAE-cellulose DE-52 column (2.5 × 37 cm, HCO_3^- form) and eluted with a 1700-mL linear gradient of 0.1–0.75 M ammonium bicarbonate. There were 170 fractions (10 mL each) collected with App(s)A (S_P) eluting in fractions 51–56 and App(s)A (R_P) eluting in fractions 57–68. The fractions containing the individual diastereomers were pooled separately, treated with a 2-fold molar excess of triethylamine, and evaporated. The residues were evaporated several more times with water before being dissolved in water to give 5 mM solutions of each diastereomer: yield App(s)A (S_P) 78 μmol (16%), App(s)A (R_P) 87 μmol (17%), total App(s)A (S_P and R_P) 165 μmol (33%).

Analysis for App(s)A (S_P): TLC R_f 0.46, DQC positive; UV (pH 7) λ_{max} 259 nm; ^{31}P NMR (D_2O) δ (PS) 43.3, (PO) −11.9 (J_{PS-PO} = 27.7 ± 2 Hz). Venom phosphodiesterase digestion of App(s)A (S_P) resulted in the production of equal amounts of AMP and AMPS as estimated by TLC analysis. The reaction solution consisted of 0.2 M Tris-HCl buffer (pH 8.8), 4.0 mM App(s)A (S_P), and 0.15 unit/mL venom phosphodiesterase in a total volume of 100 μL. The reaction solution was incubated for 3 h at 37 °C. Analysis for App(s)A (R_P): TLC R_f 0.43, DQC positive; UV (pH 7) λ_{max} 259 nm; ^{31}P NMR (D_2O) δ (PS) 43.6, (PO) −11.9 (J_{PS-PO} = 27.7 ± 2 Hz). Venom phosphodiesterase digestion of App(s)A (R_P) resulted in the production of equal amounts of AMP and AMPS as estimated by TLC analysis. The reaction conditions were the same as for App(s)A (S_P).

(B) Inosine(5')-α-thiodiphospho(5')adenosine (**3b**). Inosine 5'-phosphorothioate (IMPS) was prepared enzymatically from AMPS by a modification of the method of Murray & Atkinson (1968). The reaction solution consisted of 16.7 mM AMPS (disodium salt), 13 mM potassium cacodylate buffer (pH 6.5), 170 mM KCl, 0.33 mM β-mercaptoethanol, and 0.37 unit/mL adenylic acid deaminase in a total volume of 30 mL. After incubation for 24 h at room temperature, TLC analysis of the reaction solution showed that the AMPS had been completely converted to IMPS (R_f 0.52, DQC positive). The solution was applied to a DEAE-cellulose DE-52 column (2.7 × 28 cm, HCO_3^- form) and eluted with a 500-mL linear gradient of 0–0.4 M ammonium bicarbonate. There were 76 fractions (7 mL each) collected with IMPS eluting in fractions 54–76. The fractions containing IMPS were pooled, treated with a 2-fold molar excess of triethylamine, and evaporated. The residue was evaporated several more times with water. The yield of IMPS was 500 μmol (100%).

IMPS (500 μmol) was dissolved in water (5 mL), and the solution was passed through a Dowex 50X4 column (1.2 × 12 cm, pyridinium form). The eluant was evaporated to give the pyridinium salt of IMPS. The IMPS was then coupled with AMP by a procedure identical with that described for AMPS in the synthesis of App(s)A. The crude reaction product was dissolved in water (50 mL), and the solution was adjusted to pH 9 with triethylamine.

The aqueous solution was applied to a DEAE-cellulose DE-52 column (2.5 × 35 cm, HCO_3^- form) and eluted with a 1500-mL linear gradient of 0.1–0.5 M ammonium bicarbonate. There were 180 fractions collected (9 mL each) with App(s)I (S_P + R_P) eluting as partially resolved isomers in fractions 63–90. These fractions were pooled, treated with a 2-fold molar excess of triethylamine, and evaporated. The yield of App(s)I (S_P + R_P) was 225 μmol (45%).

In order to separate App(s)I (S_P) from App(s)I (R_P), we rechromatographed a solution of App(s)I (S_P and R_P) (30 μmol/5 mL of H_2O) on a second DEAE-cellulose DE-52 column (2.5 × 40 cm, HCO_3^- form) using a 2000-mL linear gradient of 0.1–0.7 M ammonium bicarbonate. There were 230 fractions (9 mL each) collected with App(s)I (S_P) eluting in fractions 52–59 and App(s)I (R_P) eluting in fractions 61–67. The fractions containing the individual diastereomers were pooled separately, treated with a 2-fold molar excess of triethylamine, and evaporated. The residues were evaporated several more times with water before being dissolved in water to give 10 mM solutions of each diastereomer.

Analysis for App(s)I (S_P): TLC R_f 0.56, DQC positive; UV (pH 7) λ_{max} 252 nm (ε = 21 300); ^{31}P NMR (D_2O) δ (PS) 44.0, (PO) −11.2 (J_{PS-PO} = 28.5 ± 1 Hz). Venom phosphodiesterase digestion of App(s)I (S_P) resulted in the production of AMP and IMPS as detected by TLC analysis. The reaction conditions were those previously described for App(s)A. Analysis for App(s)I (R_P): TLC R_f 0.54, DQC positive; UV (pH 7) λ_{max} 252 nm (ε = 21 300); ^{31}P NMR (D_2O) δ (PS) 44.2, (PO) −11.2 (J_{PS-PO} = 28.5 ± 1 Hz). Venom phosphodiesterase digestion of App(s)I (R_P) resulted in the production of AMP and IMPS as detected by TLC analysis. The reaction conditions were those previously described for App(s)A.

The following procedure was used to assign absolute configurations to the separated diastereomers of App(s)I. Solutions of App(s)I (S_P) and App(s)I (R_P) (1.0 μmol/0.5 mL of H_2O) were prepared, and sodium metaperiodate (1.0 μmol) was added. After a 10-min reaction period at room temperature, the reactions were quenched with β-mercaptoethanol (20 μmol). The solutions were adjusted to pH 10.5 by the dropwise addition of 0.1 N sodium hydroxide and were then heated at 50 °C for 30 min. The solutions were allowed to cool and were then applied to DEAE-Sephadex A-25 columns (0.5 × 25 cm, HCO_3^- form). The columns were eluted with 200-mL linear gradients of 0.1–0.85 M triethylammonium bicarbonate. The IDPαS-containing fractions were identified by virtue of their characteristic λ_{max} at 247 nm. These fractions were pooled and evaporated. After an additional evaporation with water, the two IDPαS residues were dissolved in water to give 0.1 mM solutions as estimated by A_{247}. Each sample of IDPαS was assayed for reactivity in a pyruvate kinase assay. The assay solutions consisted of 83 mM tri-

ethanolamine buffer (pH 7.6), 0.2 mM NADH, 0.5 mM PEP, 0.06–0.07 mM IDPαS, 125 units/mL lactic dehydrogenase, and 95 units/mL pyruvate kinase in a total volume of 1.00 mL. The assay reactions were followed spectrophotometrically by monitoring the disappearance of NADH at 340 nm (*Biochemical Information*, 1973).

(*C*) *Adenosine 2',3'-Phosphate 5'-Phosphorothioate* (**5**). Adenosine 2',3'-phosphate (**4**) was prepared from adenosine 2'(3')-phosphate according to the method of Shugar (1967). A suspension of adenosine 2',3'-phosphate (150 μmol, ammonium salt) in triethyl phosphate (12.5 mL) was cooled to 0 °C, and thiophosphoryl chloride (0.625 mL, 6.75 mmol) was added. The mixture was stirred for 17 h at 0 °C, resulting in complete dissolution. Excess thiophosphoryl chloride was removed by rotary evaporation (20 min), and ice-cold 1 M triethylammonium bicarbonate (100 mL) was added.

The solution was applied to a DEAE-cellulose DE-52 column (2.5 × 40 cm, HCO_3^- form) and eluted with a 2000-mL linear gradient of 0.05–0.4 M ammonium bicarbonate. There was 210 fractions (10 mL each) collected with p(s)A>p eluting in fractions 122–160. These fractions were pooled and concentrated to a small volume (10 mL). The solution was rechromatographed on a second DEAE-cellulose DE-52 column (1.5 × 20 cm, HCO_3^- form) with an 800-mL linear gradient of 0–0.8 M triethylammonium bicarbonate. There were 180 fractions (4.5 mL each) collected with cyclic p(s)Ap eluting in fractions 113–135. These fractions were pooled and evaporated. The residue was redissolved in water to give a 10 mM solution of p(s)A>p. The yield of p(s)A>p was 32 μmol (21%).

Analysis: TLC R_f 0.48, DQC positive [with minor spots at R_f 0.41 and 0.33, both DQC positive (2'- and 3'-p(s)Ap)]; UV (pH 7) λ_{max} 258 nm; ^{31}P NMR (D_2O) δ (PS) 43.2, (PO) 19.2. Ribonuclease T_2 hydrolysis of p(s)A>p resulted in its complete conversion to 3'-p(s)Ap as detected by TLC analysis, R_f 0.33, DQC positive. The reaction solution consisted of 6 mM p(s)A>p, 20 mM sodium citrate buffer (pH 4.6), and 200 units/mL ribonuclease T_2 in a total volume of 25 μL (Sato et al., 1966). The reaction solution was incubated for 8 h at room temperature.

(*D*) *Adenosine 2'(3')-Phosphate 5'-Phosphorothioate* (**6a,b**). p(s)A>p was subjected to mild acid hydrolysis to give p(s)Ap (2' and 3'). The reaction solution consisted of 10 mM p(s)A>p in 0.1 N HCl (1.0 mL). The reaction solution was incubated for 2 h at room temperature. The solution was adjusted to pH 8 by the dropwise addition of 3 N ammonium hydroxide. The solution was applied to a DEAE-Sephadex A-25 column (1.5 × 20 cm, HCO_3^- form) and eluted with a 500-mL linear gradient of 0–0.9 M triethylammonium bicarbonate. There were 100 fractions collected with p(s)Ap (2' and 3') eluting in fractions 74–85. These fractions were pooled and evaporated. The residue was evaporated several more times with water before being dissolved in water to give a 10 mM solution of p(s)Ap (2' and 3'). The yield of p(s)Ap (2' and 3') was 9.4 μmol (94%) in a 1:1 ratio.

Analysis: TLC R_f 0.33, DQC positive (3' isomer), 0.41, DQC positive (2' isomer); UV λ_{max} 258 nm. Alkaline phosphatase digestion of p(s)Ap (2' and 3') resulted in its complete conversion to AMPS as detected by TLC analysis, R_f 0.46, DQC positive. The reaction solution consisted of 5 mM p(s)Ap (2' and 3'), 0.5 M Tris-HCl buffer (pH 8.8), and 30 units/mL alkaline phosphatase in a total volume of 30 μL. The reaction solution was incubated for 1 h at room temperature.

Methods. (*A*) *RNA Ligase Catalyzed Reaction of App(s)A with ApApA.* The reaction solution consisted of 50 mM Hepes buffer (pH 8.3), 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, 0.5 mM ApApA, 1.5 mM App(s)A (S_P and R_P), and 115 units/mL RNA ligase in a total volume of 0.300 mL. The reaction solution was incubated for 4 h at 37 °C. The reaction solution was then applied to a DEAE-Sephadex A-25 column (0.5 × 25 cm, HCO_3^- form) and eluted with a 200-mL linear gradient of 0.1–0.7 M triethylammonium bicarbonate. There were 117 fractions (1.7 mL each) collected with the major reaction product, ApApApA, eluting in fractions 63–77, well separated from unreacted ApApA and App(s)A (S_P and R_P) in fractions 42–56. The fractions containing ApApApA were pooled and evaporated. The residue was evaporated again with water before being dissolved in water (0.050 mL). The yield of ApApApA was 0.059 μmol (39%).

Analysis: TLC R_f 0.40, DQC negative; UV (pH 7) λ_{max} 258 nm. Spleen phosphodiesterase digestion of ApApApA resulted in the production of 3'-AMP and adenosine as detected by TLC analysis, the spot pattern being identical with that obtained by spleen phosphodiesterase digestion of an ApApA standard. The reaction solution consisted of 0.1 M potassium phosphate buffer (pH 6.5), 3 mM ApApApA, and 20 units/mL spleen phosphodiesterase in a total volume of 20 μL. The reaction solution was incubated for 3 h at 37 °C.

(*B*) *RNA Ligase Catalyzed Reaction of App(s)I with ApApA.* App(s)I (S_P) and App(s)I (R_P) were individually incubated with ApApA in the presence of RNA ligase. The reaction solutions consisted of 50 mM Hepes buffer (pH 8.3), 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, 0.5 mM ApApA, 1.5 mM App(s)I (S_P or R_P), and 130 units/mL RNA ligase in a total volume of 0.300 mL. The reaction solutions were incubated for 12 h at 37 °C. The reaction solutions were then applied to DEAE-Sephadex A-25 columns (0.5 × 25 cm, HCO_3^- form) and eluted with a 250-mL linear gradient of 0.1–0.9 M triethylammonium bicarbonate. In the App(s)I (R_P) reaction, there were 160 fractions (1.6 mL each) collected with no major products eluting after ApApA in fractions 42–52 and App(s)I (R_P) in fractions 63–76. In the App(s)I (S_P) reaction, there were 160 fractions (1.6 mL each) collected with the major reaction product, ApApAp(s)I, eluting in fractions 103–117, well separated from unreacted ApApA in fractions 45–55 and unreacted App(I) (S_P) in fractions 63–78. The fractions containing ApApAp(s)I were pooled and evaporated. The residue was evaporated again with water before being dissolved in water (50 μL). The yield of ApApAp(s)I from the App(s)I (S_P) reaction was 0.040 μmol (27%).

Analysis: TLC R_f 0.37, DQC positive; UV (pH 7) λ_{max} 255 nm. Spleen phosphodiesterase digestion of ApApAp(s)I resulted in the production of 3'-AMP and a phosphodiesterase-resistant, thiophosphate-containing compound, presumably Ap(s)I, as detected by TLC analysis.[2] The reaction conditions were those previously described for the digestion of ApApApA.

In order to determine the absolute configuration of the thiophosphoryl linkage in ApApAp(s)I, we subjected a sample to venom phosphodiesterase digestion. The reaction solution consisted of 50 mM Tris-HCl buffer (pH 8.9), 0.45 mM ApApAp(s)I, and 0.55 unit/mL venom phosphodiesterase in a total volume of 20 μL. The reaction solution was incubated for 4 h at room temperature. TLC analysis of the reaction solution showed that the ApApAp(s)I was completely degraded. Under identical conditions, Ap(s)A (R_P) was also degraded whereas Ap(s)A (S_P) was unchanged.

[2] It has been shown that phosphorothioate diesters are very resistant to hydrolysis by spleen phosphodiesterase (Eckstein, 1970; Mehdi & Gerlt, 1981).

(*C*) *HPLC-Scale RNA Ligase Reactions.* Small-scale RNA ligase reactions were performed at a variety of substrate concentrations and analyzed by HPLC. The reaction solutions consisted of 50 mM Hepes buffer (pH 7.2 or pH 8.2), 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, 0.1–0.2 mM ATP, 0.1–0.2 mM p(s)Ap or pAp(s)A, 0–0.5 mM ApApA, 2.5 units/mL inorganic pyrophosphatase, and 100–200 units/mL RNA ligase in total volumes of 50–100 μL. The reaction solutions were incubated at 37 °C for various periods of time. The reaction solutions were analyzed by using an Altex Model 100 HPLC system equipped with a Whatman Partisil PXS 10/25 SAX column. The column was eluted with a linear gradient of 0.007 M KH_2PO_4, pH 4.0, to 0.25 M KH_2PO_4–0.50 M KCl, pH 4.5, for a 45-min period at a flow rate of 1.5 mL/min. The solution was monitored continuously at A_{254}. Specific reactions are discussed under Results.

(*D*) *Preparation of Adenylylated p(s)Ap.* The reaction solution consisted of 50 mM Hepes buffer (pH 7.2), 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, 0.2 mM ATP, 0.1 mM p(s)Ap (2′ and 3′), 2.5 units/mL inorganic pyrophosphatase, and 120 units/mL RNA ligase in a total volume of 1.00 mL. The reaction solution was incubated for 2 h at 37 °C. HPLC analysis of the reaction solution at this point showed the formation of a substantial amount [75% yield relative to initial 3′-p(s)Ap] of App(s)Ap (R_T = 40 min) as well as unreacted p(s)Ap (R_T = 35 min) and ATP (R_T = 46 min).

The reaction solution was diluted by the addition of 1.0 M Tris-HCl buffer (pH 8.2, 1.00 mL), and alkaline phosphatase (22 units) was added. The reaction solution was incubated for 4 h at 37 °C. HPLC analysis of the reaction solution showed the formation of App(s)A (R_T = 14 min) and adenosine (R_T = 2.4 min) as well as some unhydrolyzed App(s)Ap (R_T = 40 min).

The reaction solution was applied to a DEAE-Sephadex A-25 column (0.5 × 25 cm, HCO_3^- form) and eluted with a 200-mL linear gradient of 0.1–0.7 M ammonium bicarbonate. There were 116 fractions collected (1.7 mL each) with App(s)A eluting in fractions 44–56, well separated from adenosine in fractions 6–12 and unhydrolyzed App(s)Ap in fractions 78–90. The fractions containing App(s)A (0.037 μmol) were pooled and evaporated.

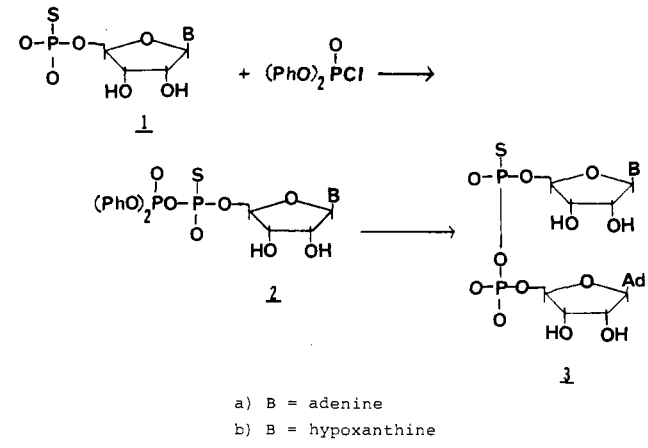
In order to determine the absolute configuration of the App(s)A, we dissolved the sample (0.037 μmol) in water (2 mL) and applied it to a second DEAE-Sephadex A-25 column (0.5 × 25 cm, HCO_3^- form) along with a sample of chemically synthesized App(s)A (R_P, 0.05 μmol). The column was eluted with a 200-mL linear gradient of 0.1–0.7 M ammonium bicarbonate. There were 116 fractions (1.7 mL each) collected with the two samples of App(s)A eluting as two partially separated peaks, the RNA ligase generated App(s)A eluting in fractions 46–55 and App(s)A (R_P) eluting in fractions 56–65.

(*E*) *Preparation of RNA Ligase Generated p(s)A>p.*[3] The reaction solution consisted of 50 mM Hepes buffer (pH 8.3), 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, 2.5 mM ATP, 1.5 mM p(s)Ap (2′ and 3′), 0.5 mM ApApA, and 490 units/mL RNA ligase in a total volume of 0.300 mL. The reaction solution was incubated for 4 h at 37 °C. HPLC analysis of the reaction solution at this point showed the formation of p(s)A>p [54% yield relative to the initial 3′-p(s)Ap concentration] (R_T = 28 min), lesser amounts (8% yield) of App(s)Ap (R_T = 40 min), and unreacted p(s)Ap (R_T = 35 min) and ATP (R_T = 46 min). The reaction solution was applied to a DEAE-Sephadex A-25 column (0.5 × 25 cm, HCO_3^- form) and eluted with a 250-mL linear gradient of 0.1–0.9 M triethylammonium bicarbonate. There were 117 fractions (2.1 mL each) collected with p(s)A>p coeluting with unreacted ATP in fractions 58–65. The fractions containing cyclic p(s)Ap were pooled and evaporated. This material was identical by TLC and HPLC with chemically synthesized p(s)A>p: TLC R_f 0.47, DQC positive; HPLC R_T = 40 min.

(*F*) *RNA Ligase Catalyzed Reaction of pAp(s)A with ApApA.* The reaction solution consisted of 50 mM Hepes buffer (pH 8.3), 20 mM $MgCl_2$, 3.3 mM DTT, 10 μg/mL BSA, 0.25 mM ATP, 0.1 mM pAp(s)A (R_P), 0.5 mM ApApA, and 25 units/mL RNA ligase in a total volume of 1.00 mL. The reaction solution was incubated for 6 h at 37 °C. The reaction solution was applied to a DEAE-Sephadex A-25 column (0.5 × 25 cm, HCO_3^- form) and eluted with a 250-mL linear gradient of 0.1–0.9 M triethylammonium bicarbonate. There were 85 fractions (3 mL each) collected with the major reaction product, ApApApAp(s)A, eluting in fractions 54–61, well separated from ADP in fractions 33–35, ATP in fractions 45–49, and unreacted ApApA in fractions 24–30. The fractions containing ApApApAp(s)A were pooled and evaporated. The residue was evaporated again with water before being dissolved in water (0.050 mL). The yield of recovered ApApApAp(s)A was 0.053 μmol (53%).

Analysis: TLC R_f 0.34, DQC positive; UV (pH 7) λ_{max} 258 nm. Spleen phosphodiesterase digestion of ApApApAp(s)A resulted in the production of 3′-AMP and a phosphodiesterase-resistant, thiophosphate-containing compound with an R_f identical with that of Ap(s)A as detected by TLC analysis.[2] The reaction conditions were those previously described for the digestion of ApApApA.

Scheme II



Results

Reactivity of App(s)A in the ATP-Independent RNA Ligase Reaction. App(s)A was prepared as a potential chiral substrate for use in stereochemical studies of the ATP-independent RNA ligase reaction. The synthesis of App(s)A is outlined in Scheme II. Adenosine was thiophosphorylated at the 5′-hydroxyl position according to the procedure of Murray & Atkinson (1968) to give AMPS (**1a**). AMPS then was treated with diphenyl phosphorochloridate to give the Michaelson-activated intermediate **2a** (Michaelson, 1964). Subsequent reaction of **2a** with AMP yielded App(s)A (**3a**). App(s)A prepared by this chemical sequence exists as an approximately equimolar mixture of two diastereomers due to the chirality of the thiophosphoryl center. DEAE-cellulose column chro-

[3] After this experiment was performed, it was found that a reaction consisting of 0.1 mM ATP, 0.2 mM p(s)Ap, and 0.5 mM ApApA at pH 7.2 results in the production of only p(s)A>p (64% yield relative to the initial ATP concentration).

matography of the reaction mixture resulted in the separation of the two diastereomers in greater than 95% isomeric purity as estimated by ^{31}P NMR analysis. The separated diastereomers were identified by ^{31}P NMR analysis, which gave a doublet at δ 44.0 for the thiophosphoryl phosphorus of the S_P isomer and a doublet at δ 44.2 for the thiophosphoryl phosphorus of the R_P isomer. Both isomers gave doublets at δ −11.2 for the phosphoryl phosphorus and an average coupling constant of J_{PS-PO} = 27.7 ± 2 Hz. These parameters compared favorably with those previously reported (Richards et al., 1978).

App(s)A (S_P and R_P) was examined as a substrate for the ATP-independent reaction catalyzed by RNA ligase. App(s)A (S_P and R_P) (1.5 mM) was incubated with the trinucleotide acceptor ApApA (0.5 mM) and RNA ligase (115 units/mL) under standard assay conditions (England et al., 1977). The reaction mixture was analyzed by DEAE-Sephadex column chromatography. The major reaction product eluted at a higher buffer concentration than ApApA, suggesting that a longer oligomer had been made. TLC analysis, however, revealed that the product did not contain a thiophosphoryl linkage. Furthermore, spleen phosphodiesterase digestion of the product resulted in the formation of only 3′-AMP and adenosine. These results indicated that an AMP unit rather than an AMPS unit had been transferred from App(s)A (S_P and R_P) to the 3′-hydroxyl terminus of ApApA to produce the tetranucleotide ApApApA as the ligation product.

Reactivity of App(s)I in the ATP-Independent RNA Ligase Reaction. App(s)I was prepared as an alternate chiral substrate for use in stereochemical studies of the ATP-independent RNA ligase reaction as outlined in Scheme II. IMPS (**1b**) was prepared from AMPS (**1a**) with adenylic acid deaminase (Murray & Atkinson, 1968). IMPS was then treated with diphenyl phosphorochloridate to give the Michaelson-activated intermediate (**2b**). Subsequent reaction of **2b** with AMP yielded App(s)I (**3b**). As with App(s)A, App(s)I prepared by this chemical sequence exists as an approximately equimolar mixture of two diastereomers due to the chirality of the thiophosphoryl center. These diastereomers were separated by DEAE-cellulose column chromatography in greater than 95% isomeric purity as estimated by ^{31}P NMR.

The absolute configuration of the separated diastereomers was established via the reaction sequence illustrated in Scheme III. A sample of each diastereomer of App(s)I was oxidized with periodate and then treated with alkali to give an equimolar mixture of ADPβS and IDPαS. The IDPαS derived from each sample was isolated by DEAE-Sephadex column chromatography and assayed for reactivity in a pyruvate kinase assay. The IDPαS derived from one diastereomer was found to be reactive in this assay whereas the IDPαS derived from the other diastereomer was unreactive. Since the pyruvate kinase reaction is stereoselective for nucleoside α-thiodiphosphates having the S_P configuration (Eckstein & Goody, 1976), the diastereomer of App(s)I that yielded pyruvate kinase reactive IDPαS was assigned the S_P configuration and the other the R_P configuration.[4] ^{31}P NMR analysis of the diastereomers gave a doublet at δ 43.3 for the thiophosphoryl phosphorus of the S_P isomer and a doublet at δ 43.6 for the thiophosphoryl of the R_P isomer. Both isomers gave doublets at δ −11.9 for the phosphoryl phosphorus and an average coupling constant of J_{PS-PO} = 28.5 ± 1 Hz. The relative chemical shifts of the S_P and R_P isomers of App(s)I are the same as was observed for the S_P and R_P isomers of App(s)A.

Scheme III

$$\text{App(s)I } (S_P) \xrightarrow[OH^-]{IO_4^-} \text{ADP}\beta\text{S} + \text{IDP}\alpha\text{S } (S_P) \xrightarrow{\text{pyruvate kinase}} \text{reactive}$$

$$\text{App(s)I } (R_P) \xrightarrow[OH^-]{IO_4^-} \text{ADP}\beta\text{S} + \text{IDP}\alpha\text{S } (R_P) \xrightarrow{\text{pyruvate kinase}} \text{unreactive}$$

The S_P and R_P isomers of App(s)I were examined as substrates for the ATP-independent reaction catalyzed by RNA ligase. Each diastereomer (1.5 mM) was incubated separately with the trinucleotide acceptor ApApA (0.5 mM) and RNA ligase (130 units/mL) under standard assay conditions. The reaction mixtures were analyzed by DEAE-Sephadex column chromatography. The App(s)I (R_P) reaction resulted in no major reaction products. The App(s)I (S_P) reaction, however, produced a major reaction product that eluted at a higher buffer concentration than ApApA, suggesting that a longer oligomer had been made. TLC analysis revealed that the product did contain a thiophosphoryl linkage. Spleen phosphodiesterase digestion of the product resulted in the formation of 3′-AMP and a phosphodiesterase-resistant, thiophosphate-containing compound, presumably Ap(s)I.[2] The UV spectrum of the product had a maximum absorbance at 255 nm, consistent with that predicted for a tetranucleotide comprised of three adenosine units and one inosine unit.[5] These results indicated that App(s)I (S_P), but not App(s)I (R_P), had under gone an RNA ligase catalyzed reaction in which an IMPS unit had been transferred from App(s)I (S_P) to the 3′-hydroxyl terminus of ApApA to produce the tetranucleotide ApApAp(s)I as the ligation product.

In order to establish the absolute configuration at the thiophosphoryl linkage of ApApAp(s)I, we subjected a sample to venom phosphodiesterase digestion. Venom phosphodiesterase catalyzes the stereoselective hydrolysis of phosphorothioate substrates having the R_P configuration (Bryant & Benkovic, 1979; Burgers & Eckstein, 1978). Under conditions where Ap(s)A (R_P) was completely digested but Ap(s)A (S_P) was resistant, the RNA ligase generated ApApAp(s)I was completely digested. The ApApAp(s)I was therefore assigned the R_P configuration.

Reactivity of p(s)Ap in the ATP-Dependent RNA Ligase Reaction. p(s)Ap was prepared as a potential prochiral substrate for use in stereochemical studies of the ATP-dependent RNA ligase reaction. The synthesis of p(s)Ap is outlined in Scheme IV. Adenosine 2′,3′-phosphate (**4**) was prepared by dicyclohexylcarbodiimide-mediated cyclization of adenosine 2′(3′)-phosphate as described by Shugar (1967). Adenosine 2′,3′-phosphate was thiophosphorylated at the 5′-hydroxyl position with thiophosphoryl chloride to give p(s)A>p (**5**). Mild acid hydrolysis of **5** resulted in the opening of the cyclic phosphate ring, yielding a mixture of 2′- and 3′-p(s)Ap (**6a,b**).

p(s)Ap was examined as a substrate for the ATP-dependent

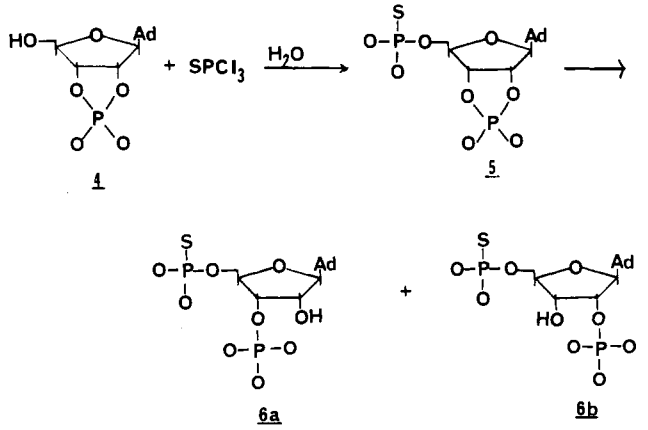
[4] These assignments are based on the presumption that the substitution of inosine for adenosine in the base portion of the nucleoside α-thiodiphosphate does not alter the stereoselectivity of the pyruvate kinase reaction.

[5] The expected λ_{max} for ApApAp(s)I at pH 7 is given by

$$\lambda_{max} = \frac{(3 \times \lambda_{max}^{Ado} \epsilon_{\lambda_{max}^{Ado}}) + (1 \times \lambda_{max}^{Ino} \epsilon_{\lambda_{max}^{Ino}})}{(3 \times \epsilon_{\lambda_{max}^{Ado}}) + (1 \times \epsilon_{\lambda_{max}^{Ino}})}$$

where λ_{max}^{Ado} = 257 nm, $\epsilon_{\lambda_{max}^{Ado}}$ = 15 300, λ_{max}^{Ino} = 247 nm, and $\epsilon_{\lambda_{max}^{Ino}}$ = 12 300.

Scheme IV



adenylylation reaction catalyzed by RNA ligase, initially in the absence of a 3′-hydroxyl acceptor. p(s)Ap (2′ and 3′, 0.1 mM) was incubated with ATP (0.2 mM) and RNA ligase (200 units/mL) under assay conditions known to favor the formation of the adenylylated intermediate (pH 7.2) (Uhlenbeck & Cameron, 1977). After 2 h of incubation time, HPLC analysis of the reaction solution showed the formation of a substantial amount [75% yield relative to the initial 3′-p(s)Ap concentration] of a new reaction product (R_T = 40 min) as well as minor amounts of ADP. When alkaline phosphatase was added to the reaction solution, all of the unreacted p(s)Ap and ATP was hydrolyzed to adenosine, and the major reaction product was converted to a new product having a retention time identical with that of App(s)A (R_T = 14 min). These results indicated that the original product of the adenylylation reaction was App(s)Ap and that this compound was dephosphorylated by the action of alkaline phosphatase to give App(s)A.

The adenylylation reaction sequence was repeated on a larger scale, and the dephosphorylated reaction product was isolated by DEAE-Sephadex column chromatography. This material was identical with App(s)A by TLC and HPLC analysis, and its DEAE-Sephadex elution position corresponded to App(s)A (S_P). When the enzymatically generated App(s)A was rechromatographed on a DEAE-Sephadex column with an equal amount of chemically synthesized App(s)A (R_P), two partially separated peaks were obtained, thus confirming that the enzymatically generated App(s)A had the S_P configuration. Since the alkaline phosphatase catalyzed dephosphorylation reaction presumably should not affect the stereochemistry of the pyrophosphate linkage, it follows that the original RNA ligase generated adenylylated intermediate, App(s)Ap, also had the S_P configuration.

p(s)Ap was next examined as a substrate for the adenylylation reaction in the presence of the 3′-hydroxyl acceptor ApApA. Initially, small-scale reactions were carried out at varied substrate concentrations and were analyzed by HPLC.[6] p(s)Ap (2′ and 3′, 0.1 mM) was preincubated for 2 h with ATP (0.2 mM) and RNA ligase (200 units/mL) to allow the accumulation of the adenylylated intermediate App(s)Ap as detected by HPLC analysis [75% yield relative to the starting 3′-p(s)Ap concentration]. ApApA (0.25 mM) and additional RNA ligase (100 units/mL) then were added, and the incubation was continued. After 2 h more, HPLC analysis showed that the App(s)Ap level was not changed and that a new unidentified product (R_T = 28 min) had formed. Similar results were obtained when the same experiment was performed with the concentrations of p(s)Ap and ATP reversed. When p(s)Ap (2′ and 3′, 0.2 mM) was incubated with limiting ATP (0.1 mM) and RNA ligase (200 units/mL), with ApApA (0.5 mM) present from the beginning, only the unidentified product was formed (64% yield relative to initial ATP concentration).[3] A variation of the first reaction (see Experimental Procedures) was repeated on a larger scale, and the unidentified product was purified by DEAE-Sephadex column chromatography. This product was shown to be identical by TLC and HPLC analysis with p(s)A>p.

Reactivity of pAp(s)A in the ATP-Dependent RNA Ligase Reaction. The R_P and S_P isomers of pAp(s)A were examined as donor substrates for the ATP-dependent RNA ligase reaction. The adenylylation reaction was initially carried out in the absence of a 3′-hydroxyl acceptor. Each diastereomer (0.1 mM) was incubated separately with ATP (0.2 mM) and RNA ligase (100 units/mL) under assay conditions known to favor the formation of the adenylylated intermediate (pH 7.2). After 3 h of incubation time, HPLC analyses of the two reaction solutions were very similar, showing the formation of a new reaction product [R_T = 22 min (S_P) and 23 min (R_P)] as well as minor amounts of ADP. After 20 h, there were substantial amounts (50–60% yield) of this product formed in each reaction solution. These results indicated that both isomers of pAp(s)A had undergone an RNA ligase catalyzed reaction with ATP to give the adenylylated intermediate AppAp(s)A.

R_P and S_P pAp(s)A were next employed as substrates for the ATP-dependent RNA ligase reaction in the presence of the 3′-hydroxyl acceptor ApApA. Each diastereomer (0.1 mM) was incubated separately with ATP (0.25 mM), ApApA (0.5 mM), and RNA ligase (100 units/mL) under assay conditions known to favor the formation of ligated products (pH 8.3) (Uhlenbeck & Cameron, 1977). After 5 h of incubation time, HPLC analyses of the two reaction solutions were identical, each showing the formation of a new reaction product (R_T = 56 min) as well as minor amounts of ADP. There was no trace of unreacted pAp(s)A or adenylylated intermediate, AppAp(s)A, in either reaction solution, indicating that both isomers of pAp(s)A had been completely converted to the ligated product ApApApAp(s)A.

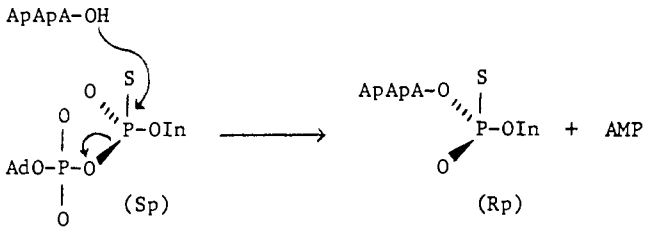
The pAp(s)A (R_P) reaction was repeated on a larger scale and the reaction product was isolated by DEAE-Sephadex column chromatography. The product eluted at a higher buffer strength than ApApA, suggesting that a longer oligomer had been made. TLC analysis revealed that the product did contain a thiophosphoryl linkage. Spleen phosphodiesterase digestion of the product resulted in the formation of 3′-AMP and a phosphodiesterase-resistant, thiophosphate-containing compound having an R_f identical with that of Ap(s)A, as detected by TLC analysis. These results indicated that the product most likely was the expected pentanucleotide ApApApAp(s)A.

Discussion

App(s)A (S_P and R_P) was found to be a reactive substrate in the ATP-independent reaction when incubated with the 3′-hydroxyl acceptor ApApA and RNA ligase. However, the reaction occurred at the phosphoryl center rather than at the chiral thiophosphoryl center, resulting in the transfer of an AMP unit from App(s)A to ApApA to produce the tetranucleotide ApApApA as the primary ligation product. This suggests that any preference for AMP over AMPS as the leaving group in the ATP-independent reaction is not sufficient

[6] The RNA ligase preparation contained an activity that degraded ApApA: RNA ligase at 130 units/mL degraded 0.5 mM ApApA 5–10% in 8 h (pH 8.3) as detected by HPLC analysis.

Scheme V



to overcome the expected lower reactivity of the thiophosphoryl center.

When App(s)I was incubated with ApApA and RNA ligase in an ATP-independent reaction, it was found that the S_P isomer, but not the R_P isomer, was a reactive substrate. With the S_P isomer, reaction occurred at the chiral thiophosphoryl center, resulting in the transfer of the IMPS unit from App(s)I to ApApA to produce the tetranucleotide ApApAp(s)I as the primary ligation product. In this case, the reaction was forced to occur at the thiophosphoryl center because reaction at the phosphoryl center would require the highly unfavorable expulsion of IMPS as the leaving group. The reaction at the chiral thiophosphoryl center also is evident in the isomeric discrimination exhibited by RNA ligase between the two diastereomeric forms of the substrate.

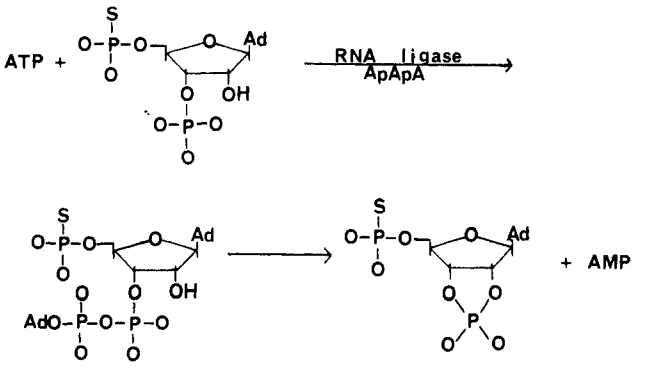
The absolute configuration of the thiophosphodiester bond in the ligated product was determined to be R_P by virtue of its reactivity toward venom phosphodiesterase. Since the ligated product was formed from App(s)I having the S_P configuration, the RNA ligase catalyzed IMPS group transfer occurs with overall inversion of configuration. Recent studies indicate that each individual enzymatic displacement step at phosphorus proceeds with inversion stereochemistry (Knowles, 1980). Overall inversion stereochemistry at phosphorus in an enzymatic reaction therefore implies the occurrence of a single or odd number of displacement steps in the reaction mechanism. In the case of the ATP-independent RNA ligase reaction, the observation of overall inversion stereochemistry suggests that the reaction most likely involves a direct displacement of AMP from App(s)I by the 3′-hydroxyl group of ApApA, to give thiophosphodiester bond formation (Scheme V). Presumably, the phosphodiester bond-forming step of the ATP-dependent RNA ligase reaction similarly proceeds by a direct displacement mechanism with inversion stereochemistry.[7]

With the stereochemical course of the phosphodiester bond-forming step established, it became of interest to determine if RNA ligase catalyzed adenylylation of a prochiral phosphorothioate substrate would result in the stereospecific formation of a chiral adenylylated intermediate. For this study, p(s)Ap was prepared as a prochiral analogue of the minimal donor substrate utilized by RNA ligase in the ATP-dependent reaction.

Incubation of p(s)Ap with ATP and RNA ligase in the absence of 3′-hydroxyl acceptor resulted in the accumulation of the expected adenylylated intermediate, App(s)Ap. The absolute configuration of this intermediate was shown to be S_P by removing the 3′-phosphoryl group with alkaline phosphatase and comparing the resulting App(s)A chromatographically to chemically synthesized S_P and R_P App(s)A.

[7] The stereochemical consequences of the reactions catalyzed by seven enzymes have been determined with both chiral oxygen and phosphorothioates; in each case, the stereochemical outcomes obtained from the two approaches were identical, suggesting that sulfur substitution is not expected to alter the stereochemical course of enzyme-catalyzed displacement reactions at phosphorus (Mehdi & Gerlt, 1981).

Scheme VI



Thus, RNA ligase catalyzed adenylylation of p(s)Ap does result in the production of a chiral intermediate having the stereochemical configuration required for the subsequent phosphodiester bond-forming step.

When p(s)Ap was incubated with ATP and RNA ligase in the presence of the 3′-hydroxyl acceptor ApApA in an effort to couple the adenylylation and phosphodiester bond-forming steps of the ATP-dependent reaction, none of the expected ligation product, ApApAp(s)Ap, and little or none (depending on the exact reaction conditions) of the adenylylated intermediate, App(s)Ap, were formed. Instead, the major product of the reaction was p(s)A>p. A plausible mechanism for the formation of this unexpected product is illustrated in Scheme VI. The first step of this mechanism requires an RNA ligase catalyzed adenylylation of the 3′-phosphoryl group of p(s)Ap to give the 3′–5′ phosphoanhydride derivative p(s)AppA. Precedent for proposing such a 3′-phosphoryl adenylylation reaction stems from the studies of Gumport et al. (1980), who have observed that at high ATP concentrations, RNA ligase will catalyze the adenylylation of the 3′-phosphoryl group of oligodeoxyribonucleotides owing to a second mode of binding. Incorrect binding in the case of p(s)Ap may occur as a result of the sulfur substitution of the 5′-phosphoryl group since the analogous nucleoside bisphosphate pAp is correctly adenylylated at the 5′-phosphoryl group and can be ligated to a variety of 3′-hydroxyl acceptors (England & Uhlenbeck, 1978; Kikuchi et al., 1978). Why RNA ligase should correctly adenylylate the 5′-thiophosphoryl group of p(s)Ap in the absence of ApApA but incorrectly adenylylate the 3′-phosphoryl group in the presence of ApApA is unclear. Equally perplexing is the inability of the ligase to convert App(s)Ap to product in the presence of the acceptor ApApA.

The second step of the proposed mechanism for the formation of p(s)A>p invokes spontaneous cyclization of the adenylylated intermediate due to nonenzymatic intramolecular nucleophilic attack by the 2′-hydroxyl group on the activated 3′-phosphoryl group. Intramolecular cyclization reactions have been observed in the attempted preparation of oligoribonucleotides having activated 3′-phosphoryl groups (Gumport et al., 1980); thus, it is reasonable to expect that the proposed activated 3′-phosphoryl intermediate p(s)AppA would similarly cyclize to the observed reaction product p(s)A>p. More recently, it has been reported that when a 3′-phosphate terminated oligoribonucleotide is incubated with ATP in the presence of RNA ligase, a low yield of the cyclic 2′,3′-phosphate terminated oligomer is obtained. A similar adenylylation–cyclization mechanism was also proposed to explain these results (Hinton et al., 1982). The ability of RNA ligase to generate cyclic 2′,3′-phosphate, although amplified by the thiophosphoryl moiety, may be an important function and is akin to the formation of a 2′-phosphomonoester 3′,5′-

phosphodiester linkage catalyzed by an RNA ligase in wheat germ in a series of steps; one of which may involve a cyclic 2′,3′-phosphate (Konarska et al., 1981).

The R_P and S_P isomers of pAp(s)A were examined as examples of donor substrates having chiral thiophosphoryl linkages adjacent to the 5′-phosphoryl group undergoing the ligation reaction. Both isomers were found to readily undergo the ATP-dependent adenylylation reaction as well as ligation to the acceptor trinucleotide ApApA. The chirality of the thiophosphoryl center appeared to have little effect on either the adenylylation or ligation reaction. Although the reactions of these substrates do not provide additional stereochemical information regarding the catalytic mechanism of RNA ligase, they do demonstrate the feasibility of introducing thiophosphoryl linkages of either the R_P or S_P configuration into RNA chains. This approach avoids the problems of reduced reactivity and stereospecificity that may limit the usefulness of compounds having the general structure A-5′pp(s)5′-A as synthetic reagents.

Acknowledgments

We thank Professors O. Uhlenbeck and R. Gumport for their advice and generous gifts of enzymes.

References

Biochemical Information (1973) Vol. I, Biochemica, Boehringer Mannheim Gmbh.

Bryant, F. R., & Benkovic, S. J. (1979) *Biochemistry 18*, 2825–2828.

Bryant, F. R., & Benkovic, S. J. (1981) *J. Am. Chem. Soc. 103*, 696–697.

Burgers, P. M. J., & Eckstein, F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4798–4800.

Cranston, J. W., Silber, R., Malathi, V. G., & Hurwitz, J. (1974) *J. Biol. Chem. 249*, 7447–7456.

Eckstein, F. (1970) *J. Am. Chem. Soc. 92*, 4718–4723.

Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685–1691.

England, T. E., & Uhlenbeck, O. C. (1978) *Biochemistry 17*, 2069–2076.

England, T. E., Gumport, R. I., & Uhlenbeck, O. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4839–4842.

Gordon, A. J., & Ford, R. A. (1972) *The Chemist's Companion*, p 433, Wiley, New York.

Gumport, R. I., Hinton, D. M., Pyle, V. S., & Richardson, R. W. (1980) *Nucleic Acids Res., Spec. Publ. n7*, 167.

Hinton, D. M., Brennan, C. A., & Gumport, R. I. (1982) *Nucleic Acids Res. 10*, 1877–1894.

Kikuchi, Y., Hishinuma, F., & Sakaguchi, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1270.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.

Konarska, H., Filipowiez, W., Domday, H., & Gross, H. J. (1981) *Nature (London) 293*, 112–116.

Marlier, J. F., & Benkovic, S. J. (1980) *Tetrahedron Lett. 21*, 1121–1124.

Mehdi, S., & Gerlt, J. A. (1981) *J. Am. Chem. Soc. 103*, 7018.

Michaelson, A. M. (1964) *Biochim. Biophys. Acta 91*, 1–13.

Murray, W. A., & Atkinson, M. R. (1968) *Biochemistry 7*, 4023–4029.

Ohtsuka, E., Nishikawa, S., Sugiura, M., & Ikehara, M. (1976) *Nucleic Acids Res. 3*, 1613–1623.

Richards, J. P., Ho, H., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7756–7757.

Sato, S., Uchida, T., & Egam, F. (1966) *Arch. Biochem. Biophys. 115*, 48–52.

Shugar, D. (1967) *Methods Enzymol. 12*, 131–137.

Sninsky, J. J., Last, J. A., & Gilham, P. T. (1976) *Nucleic Acids Res. 33*, 3157–3166.

Stenerson, J. (1971) *J. Chromatogr. 54*, 77–81.

Sugino, A., Snopek, T. J., & Cozzarelli, N. R. (1977) *J. Biol. Chem. 252*, 1732–1737.

Uhlenbeck, O. C., & Cameron, V. (1977) *Nucleic Acids Res. 4*, 85–98.

Walker, G. C., Uhlenbeck, O. C., Bedows, E., & Gumport, R. I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 122–126.