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Donor Activation in the T_4 RNA Ligase Reaction

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ABSTRACT: T_4 RNA ligase catalyzes the adenylation of donor oligonucleotide substrates. These activated intermediates react with an acceptor oligonucleotide which results in phosphodiester bond formation and the concomitant release of AMP. Adenylation of the four common nucleoside 3′,5′-bisphosphates as catalyzed by T_4 RNA ligase in the absence of an acceptor oligonucleotide has been examined. The extents of product formation indicate that pCp is the best substrate in the reaction and pGp is the poorest. Kinetic parameters for the joining reaction between the preadenylated nucleoside 3′,5′-bisphosphates, A(5′)pp(5′)Cp or A(5′)pp(5′)Gp, and a good acceptor substrate (ApApA) or a poor acceptor substrate (UpUpU) have been determined. The apparent K_m values for both preadenylated donors in the joining reaction are similar, and the reaction velocity is much faster than observed in the overall joining reaction. The nonnucleotide adenylated substrate P^1-(5′-adenosyl) P^2-(*o*-nitrobenzyl) diphosphate also exhibits a similar apparent K_m but reacts with a velocity 80-fold slower than the adenylated nucleoside 3′,5′-bisphosphates. By use of preadenylated donors, oligonucleotide substrates can be elongated more efficiently than occurs with the nucleoside 3′,5′-bisphosphates.

While T_4 RNA ligase is useful in catalyzing the formation of a phosphodiester bond between an acceptor oligonucleotide and a corresponding donor (Gumport & Uhlenbeck, 1981; Uhlenbeck & Gumport, 1982) and can be used with some success with similar 2′-deoxyoligonucleotides (Hinton et al., 1978; Hinton & Gumport, 1979; McCoy & Gumport, 1980), its effectiveness is limited in that it does not work equally well with all oligonucleotide sequences (England & Uhlenbeck, 1978; Romaniuk et al., 1982). Generally acceptors with a high purine content and pyrimidine-containing donors result in the highest yields of joined product. However, acceptor molecules of the same nucleoside composition but differing sequence can nevertheless react with a common donor and result in vastly different product yields (Romaniuk et al., 1982).

The mechanism of phosphodiester bond formation as catalyzed by the enzyme is similar to that which has been elucidated for DNA ligase (Modrich & Lehman, 1973; Lehman, 1974) although the substrate specificities are different. DNA ligase requires a double-stranded substrate and RNA ligase is more active with single-stranded substrate. Three distinct and reversible steps are involved (Uhlenbeck & Cameron, 1977; Sugino et al., 1977). In the first step the enzyme reacts with ATP to form an adenylated enzyme intermediate and pyrophosphate. In the second step the enzyme binds a donor molecule containing a 5′-terminal phosphate and catalyzes the formation of the adenylated donor: A(5′)pp(5′)Np.... Subsequently, the formation of a phosphodiester bond occurs between the acceptor and activated donor with concomitant

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release of AMP. Steady-state kinetic parameters for the overall reaction have been difficult to obtain since the enzyme does not appear to become saturated with substrate and, after turning over relatively few times, loses activity. Additionally the initial velocity measurements per milligram of enzyme have been reported to depend upon enzyme concentration (McCoy & Gumport, 1980; England & Uhlenbeck, 1978). The extent of phosphodiester bond formation is thus related to enzyme concentration, and increased product yields can often be obtained by subsequent addition of enzyme when the formation of joined product appears to have ceased (Uhlenbeck & Cameron, 1977; Ohtsuka et al., 1980b). An apparent K_m of 12 μM has been reported for the first step of the reaction, the reversible formation of ATP and PP_i (Cranston et al., 1974). Kinetic parameters for the circularization reaction, where a $(pA)_n$ oligomer contains both an acceptor 3′-hydroxyl and donor 5′-terminal phosphate, have also been examined (Silber et al., 1972; Kaufman et al., 1974). Most efficient phosphodiester bond formation occurs with substrates $(pA)_{10}$ to $(pA)_{16}$.

We have examined separately the second step of the reaction, the adenylation of the donor molecule (in the absence of an acceptor molecule), and the third mechanistic step, that of phosphodiester bond formation between an adenylated donor molecule and an acceptor oligonucleotide. For the latter reaction preliminary kinetic data are reported. Additionally we wish to report that very effective polymer elongation occurs between acceptor oligonucleotides and activated donors which have been chemically synthesized.

Experimental Procedures

Materials. The four nucleoside 3′,5′-bisphosphates and the four P_1-(5′-adenosyl) P_2-nucleosidyl diphosphates were obtained from P-L Biochemicals (St. Goar, FRG). UpUpU, ApApA, bacterial alkaline phosphatase, pyruvate kinase, phosphoenolpyruvate, and snake venom phosphodiesterase were products of Boehringer (Mannheim, FRG). Polynucleotide kinase was from New England Nuclear (Dreieich, FRG). Ribonuclease T_1 and pancreatic ribonuclease were obtained from Sankyo through Koch Light Laboratories (Frankfurt, FRG). Adenosine 5′-phosphomorpholidate (*N,N*′-dicyclohexylcarboxamidine salt) was a product of Sigma (Munich, FRG).

T_4 RNA Ligase Isolation. T_4 RNA ligase was isolated by a modification of a published procedure (McCoy & Gumport, 1980). After cell lysis with the aid of a French press, the crude lysate was treated with 5 mg of deoxyribonuclease. The lysate was subsequently centrifuged, and the supernatant was adsorbed on a DEAE-Sepharose CL-6B column in 50 mM Tris-HCl,[1] pH 7.5, 10 mM $MgSO_4$, 5 mM dithiothreitol, 10 nM phenylmethanesulfonyl fluoride, and 5% glycerol and eluted with a linear gradient from 0 to 0.5 M NaCl. The fractions containing enzyme activity (0.25 M NaCl) were diluted in the starting buffer and rechromatographed on a DEAE-cellulose DE-52 column by using a 0–0.4 M NaCl linear gradient. Active fractions were precipitated in 70% ammonium sulfate and centrifuged. The protein pellet was dissolved in 0.02 M HEPES, 0.02 M KH_2PO_4, pH 7.2, 1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol and chromatographed on a Sepharose ACA 34 gel filtration column. The fractions exhibiting enzyme activity (eluting near the end of the protein profile) were adsorbed on a hydroxylapatite column equilibrated with the same buffer and eluted with a linear gradient from 0.02 to 0.12 M KH_2PO_4, pH 7.2. The active enzyme fractions (0.06 M phosphate) were reduced in volume by placing them in dialysis tubing and covering them with polyethylene glycol 6000 overnight at 4 °C and subsequently dialyzed against 25 mM Hepes, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, and 50% glycerol.

By use of $(pA)_{10}$ (3.5 μM) in the circularization assay, the specific activity of the enzyme was 1400 units/mg. This is somewhat lower than previously reported (McCoy & Gumport, 1980) and probably results from the use of the shorter circularization substrate. SDS–polyacrylamide gel electrophoresis indicated the characteristic double band for the free and adenylated enzyme (Higgins et al., 1977).

Some enzyme preparations exhibited a significant ATPase activity. In these cases a final purification step on Affi-Gel blue was done with 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 10 mM $MgSO_4$, 5% glycerol, and a linear gradient from 0 to 0.3 M NaCl. The fractions eluting at 0.15 M NaCl were reduced in volume and dialyzed as described above. This reduced the ATPase contamination and additionally resulted in pure unadenylated enzyme as analyzed by SDS–polyacrylamide gel electrophoresis.

Adenylated Donor Formation. Adenylation of donor substrates was examined in a 0.1 mL reaction volume at 37 °C containing 50 mM HEPES, pH 8.4, 3.3 mM dithiothreitol, 20 mM $MgCl_2$, 100 μg/mL T_4 RNA ligase, 1.0 mM pNp, and 3–3.5 mM ATP. Aliquots of 10 μL were removed at the desired time intervals at the reaction stopped by freezing in liquid nitrogen. Analysis was by HPLC (McLaughlin & Romaniuk, 1982).

Enzyme Kinetics. Since the extent of phosphodiester bond formation as catalyzed by T_4 RNA ligase has been reported (McCoy & Gumport, 1980; England & Uhlenbeck, 1978) to depend on enzyme concentration, initial rates of reactions involving the formation of a 3′–5′ phosphodiester bond were examined in a 0.05 mL reaction volume at 37 °C containing 50 mM HEPES, pH 8.4, 3.3 mM dithiothreitol, 20 mM $MgCl_2$, 10 μg/mL bovine serum albumin, 0.5 mM ApApA, 0.7 mM A(5′)pp(5′)Gp, and an enzyme concentration of 8, 16, 32, or 48 μg/mL. Reactions were initiated by the addition of enzyme. At 0-, 1-, 2-, and 4-min time intervals 5-μL aliquots were removed, the reaction was stopped by addition to 10 μL of 100 mM phosphate, pH 4.5, and the mixture was frozen immediately in liquid nitrogen. Analysis was by HPLC (McLaughlin & Romaniuk, 1982). The extent of reaction under these conditions was quantitative (>99%) at enzyme concentrations of 48, 32, 16, and 8 μg/mL within 60 min.

Kinetic parameters for the activated donors were obtained in duplicate from 0.1-mL reaction mixtures containing 50 mM HEPES, pH 8.4, 3.3 mM dithiothreitol, 20 mM $MgCl_2$, 10 μg/mL bovine serum albumin, 32 μg/mL T_4 RNA ligase, 0.5 mM ApApA or UpUpU, and from 0.13 to 1.0 mM A(5′)-pp(5′)Gp or A(5′)pp(5′)Cp. Reactions were initiated by the addition of enzyme. At time intervals of 0, 1, 2, 3, 4, and 5 min, 5-μL aliquots were removed, and the reaction was stopped by addition to 10 μL of 100 mM phosphate, pH 4.5, and immediate freezing in liquid nitrogen. Analysis was by HPLC (McLaughlin & Romaniuk, 1982). Kinetic parameters for the acceptor (ApApA) were obtained in duplicate under

[1] Abbreviations: A(5′)pp(5′)Cp, P^1-(5′-adenosyl) P^2-[5′-(3′-phosphorylcytidyl)] diphosphate; A(5′)pp(5′)Gp, P^1-(5′-adenosyl) P^2-[5′-(3′-phosphorylguanosyl)] diphosphate; A(5′)pp(5′)Up, P^1-(5′-adenosyl) P^2-[5′-(3′-phosphoryluridyl)] diphosphate; A(5′)pp-Bzl-NO_2, P^1-(5′-adenosyl) P^2-(*o*-nitrobenzyl) diphosphate; A(5′)pp(5′)GpU, P^1-(5′-adenosyl) P^2-[5′-(guanosyl-3′(5′)-uridyl)] diphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; ATPase, adenosinetriphosphatase; HPLC, high-performance liquid chromatography.

identical conditions with the following exceptions: Reaction mixtures contained 0.7 mM A(5′)pp(5′)Gp and from 0.2 to 1.0 mM ApApA. In all cases reactions were complete within 120 min.

Enzyme Adenylation. Adenylation of T_4 RNA ligase in the absence of a donor or acceptor substrate was done by using the buffer conditions described above with 80 μg/mL T_4 RNA ligase, 3.0 mM ATP, and additionally 25 μg/mL pyrophosphatase. After a 15-min incubation at 37 °C, A(5′)pp(5′)Gp was added to a concentration of 1.0 mM and CpApCpC to a concentration of 0.5 mM. After addition of the acceptor and adenylated donor substrate the final T_4 RNA ligase concentration was 64 μg/mL. HPLC analysis of 5-μL aliquots was made immediately after the addition of the oligonucleotide substrates and after subsequent incubation at 37 °C for 30 min. At this point pCp was added to the remaining 40-μL reaction mixture to a concentration of 1.0 mM, and HPLC analyses were again made immediately after addition of the pCp substrate and additionally after a 30-min incubation at 37 °C.

Oligonucleotide Synthesis Using RNA Ligase. Oligonucleotide synthesis reactions involving an acceptor (0.5 mM) and a preadenylated donor (0.7 mM) were done in 50 mM HEPES, pH 8.4, 3.3 mM dithiothreitol, 20 mM $MgCl_2$, and 10 μg/mL bovine serum albumin. Reactions requiring donor adenylation included ATP at a concentration of 3–3.5 mM. Analysis of the reactions was done by HPLC on an APS-Hypersil column as described elsewhere (McLaughlin & Romaniuk, 1982).

Preparation of A(5′)pp(5′)Np Intermediates. To 5 mL of a reaction mixture containing 4.8 mM GpU, 10 mM ATP, 50 mM HEPES, pH 8.0, 20 mM $MgCl_2$, 3 mM dithiothreitol, and 10 μg/mL bovine serum albumin was added 200 units of polynucleotide kinase. After incubation at 37 °C for 24 h HPLC analysis indicated that complete phosphorylation had occurred. To the reaction mixture was added 0.1 mL of 2 M KCl, 0.35 mL of 100 mM phosphoenolpyruvate, and 25 μL of pyruvate kinase (10 mg/mL). After incubation for 1 h at 37 °C HPLC analysis indicated that the ADP had been completely converted to ATP. pGpU was isolated by chromatography on DEAE-Sephadex A-25 (2.0 × 25 cm) using a gradient of 1 L each of 0.02 M and 0.5 M triethylammonium bicarbonate (TEAB), pH 7.5. The product eluted at approximately 0.35 M TEAB.

pGpU (21 μmol) was converted to its trioctylammonium salt, coevaporated 3 times from dry pyridine, and dissolved in 2 mL of dry dimethylformamide; 1.5 equiv of adenosine 5′-phosphomorpholidate (*N*,*N*′-dicyclohexylcarboxamidine salt) was coevaporated 3 times from dry pyridine, dissolved in 2 mL of dry dimethylformamide, and added to the solution of pGpU. After reaction at 50 °C for 18 h HPLC analysis indicated that 70% of the pGpU was converted to product. The dimethylformamide was evaporated and the residue dissolved in 4 mL of 0.1 M ammonium acetate, pH 8.0, and treated with 20 μL of bacterial alkaline phosphatase (11 mg/mL) for 1 h at 37 °C. HPLC analysis indicated that the remaining pGpU had been converted to GpU. Isolation was by chromatography on DEAE-Sephadex A-25 (2.0 × 25 cm) using a gradient of 1 L each of 0.02 M and 0.5 M TEAB, pH 7.5. The A(5′)pp(5′)GpU eluted at approximately 0.35 M TEAB.

A(5′)pp(5′)GpU (4.6 μmol) was treated with 50 units of ribonuclease T_1 in 10 mM sodium acetate, pH 5.1, for 18 h at 37 °C. HPLC analysis on an ion-exchange column indicated quantitative conversion to uridine and a product with a longer retention time. The product was isolated by preparative HPLC on a 9.4 × 250 mm column of ODS-Hypersil using 0.1 M triethylammonium acetate at a flow rate of 4 mL/min and a gradient from 0 to 35% acetonitrile in 30 min. The product A(5′)pp(5′)Gp eluted with a retention time of 8.5 min. After lyophilization of the volatile buffer 4.2 μmol of product was obtained. The isolated product could be quantitatively converted to 1 equiv each of adenosine 5′-phosphate and guanosine 3′,5′-bisphosphate upon treatment with snake venom phosphodiesterase.

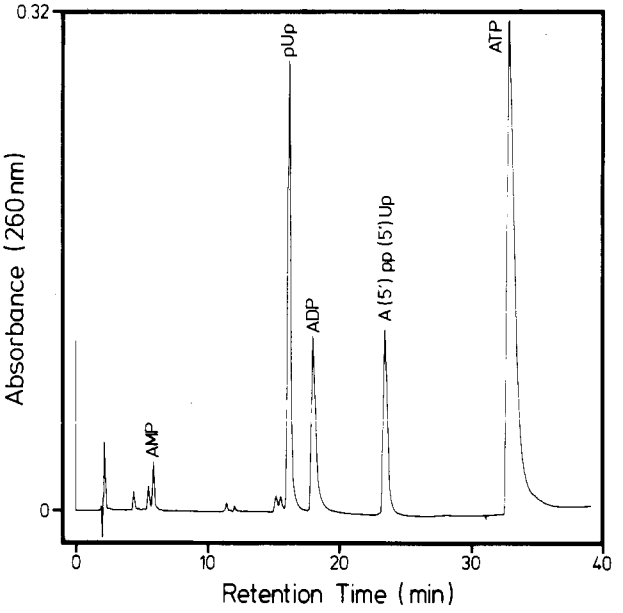

FIGURE 1: Analysis by HPLC of the adenylation of pUp after a 6-h incubation using a 4.6 × 250 mm APS-Hypersil column at 1.5 mL/min and a linear gradient from 0.05 M KH_2PO_4 to 0.9 M KH_2PO_4 in 60 min.

A(5′)pp(5′)Cp was prepared in an analogous procedure starting with CpC and using in the final step RNase A instead of RNase T_1.

RESULTS

In the absence of an acceptor and in the presence of ATP, T_4 RNA ligase catalyzed the adenylation of the 5′-phosphate of the four common nucleoside 3′,5′-bisphosphates. Analysis of the reaction mixtures after various incubation times was done by HPLC on an ion-exchange column. An example of the analysis in the conversion of pUp to A(5′)pp(5′)Up after a 6-h incubation at 37 °C is shown in Figure 1. Identification of the pyrophosphate product was done in some cases by showing that it coeluted with an authentic standard prepared by a combined chemical–enzymatic synthesis (see Experimental Procedures). Additionally the material in question could be isolated and treated with bacterial alkaline phosphatase. Samples treated in this manner coeluted with a commercially available standard of A(5′)pp(5′)N.

All enzyme preparations we have examined, both those isolated in this laboratory and those from commercial sources, generally exhibited a small ATPase activity. As a result, measurement of donor adenylation by monitoring the disappearance of ATP was difficult. However, the reaction could be monitored according to the disappearance of the pNp substrate or by directly measuring the appearance of the A(5′)pp(5′)Np product.

Under the conditions of the assay the enzyme remained active for a minimum of 6 h at 37 °C. The relative extents of adenylation of a 1.0 mM solution of the four common pNp derivatives in the presence of 100 μg/mL T_4 RNA ligase at

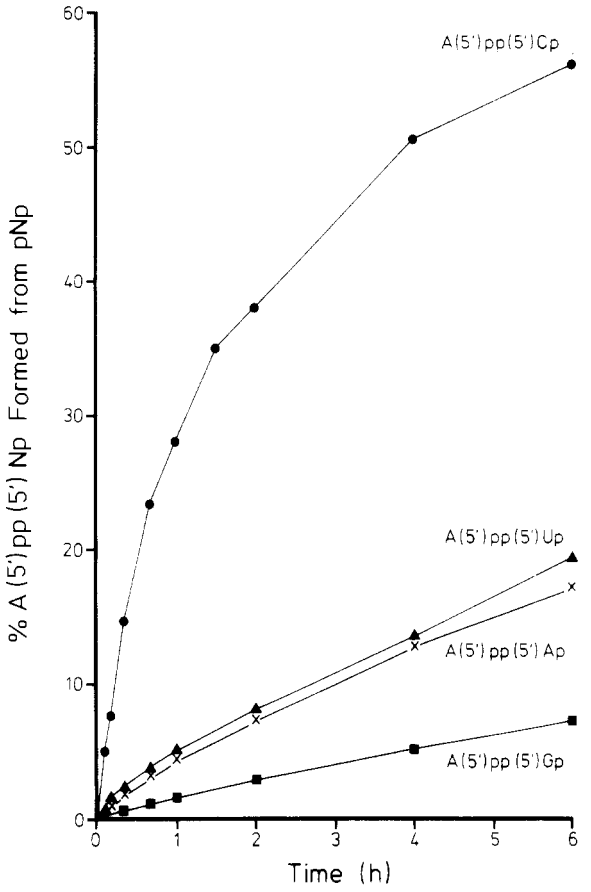

FIGURE 2: Extent of adenylation of the four common pNp substrates using 100 μg/mL T_4 RNA ligase at 37 °C in 50 mM HEPES, pH 8.4, 3.3 mM dithiothreitol, 20 mM $MgCl_2$, 10 μg/mL bovine serum albumin, 3.3 mM ATP, and 1.0 mM pNp.

37 °C during a 6-h incubation is shown in Figure 2. pCp is clearly the best substrate in the donor activation reaction. pUp and pAp are intermediate in substrate activity, and pGp is the poorest substrate resulting in only 6% product formation after a 6-h incubation. Preliminary kinetic measurements indicate that pCp exhibits an apparent K_m of about 1 mM with pGp an order of magnitude higher.

In order to measure the kinetic parameters involved in the third step of the joining reaction catalyzed by RNA ligase, it was necessary to prepare the desired activated donor molecules. Since pCp was the best and pGp the poorest substrate in the adenylation reaction, we decided to synthesize and compare the two substrates A(5′)pp(5′)Cp and A(5′)pp(5′)Gp. Both substrates were prepared by a combined chemical–enzymatic synthesis. In the first step of the synthesis CpC or GpU was phosphorylated with polynucleotide kinase. However, the dinucleoside diphosphate produced tended to coelute with ADP during isolation by DEAE-Sephadex A-25 chromatography so that pyruvate kinase and phosphoenolpyruvate had to be added to convert the ADP formed during the reaction to ATP. After chemical formation of the pyrophosphate derivative bacterial alkaline phosphatase was used to dephosphorylate the residual dinucleoside diphosphate starting material such that it did not coelute with the A(5′)pp(5′)NpN product. Cleavage of the 3′-terminal nucleoside with either RNase T_1 or pancreatic RNase was both highly specific and quantitative.

For accurate measurement of the desired kinetic parameters involving the third step of the reaction it is necessary that the unadenylated enzyme be used. Resolution of adenylated from unadenylated enzyme can be done by using SDS–polyacrylamide gel electrophoresis (Higgins et al., 1977). The enzyme used for reactions involving preadenylated donors was pure unadenylated enzyme. It could be converted in part to the adenylated form by incubation in the presence of ATP. In our hands complete adenylation of the enzyme required removal of the inorganic pyrophosphate formed with inorganic pyrophosphatase (see Experimental Procedures). That the adenylated enzyme will not use the activated donor as a substrate was shown by the following experiment. By use of ATP, T_4 RNA ligase was adenylated in the presence of pyrophosphatase (inorganic). Subsequently, an acceptor substrate (CpApCpC) and the adenylated donor A(5′)pp(5′)Gp were added. After a 30-min incubation at 37 °C no product formation was observed. To confirm that the T_4 RNA ligase was still active, pCp was then added to the reaction mixture. After an additional 30-min incubation at 37 °C the acceptor substrate was completely consumed. The ratio of the two products formed, CpApCpCpCp to CpApCpCpGp, was roughly 5 to 1.

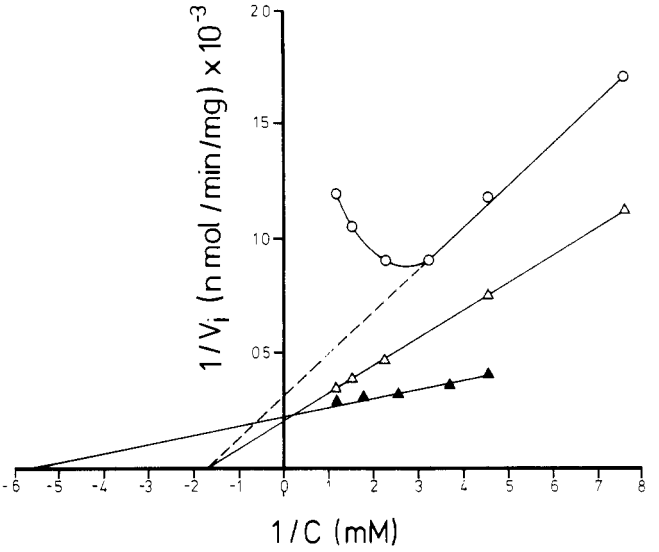

FIGURE 3: Lineweaver–Burk plot for the reaction of the preadenylated donor with suitable acceptor using 32 μg/mL T_4 RNA ligase at 37 °C in 50 mM HEPES, pH 8.4, 3.3 mM dithiothreitol, 20 mM $MgCl_2$, and 10 μg/mL bovine serum albumin. Other conditions were as in the footnotes to Table I. (○) A(5′)pp(5′)Gp in the presence of 0.5 mM UpUpU. (△) A(5′)pp(5′)Gp in the presence of 0.5 mM ApApA. (▲) ApApA in the presence of 0.7 mM A(5′)pp(5′)Gp.

Initial velocity measurements determined per milligram of enzyme at enzyme concentrations of 8, 16, 32, and 48 μg/mL for the reaction between a preadenylated donor, A(5′)pp(5′)Gp, and the acceptor, ApApA, were identical. The kinetic parameters for phosphodiester bond formation as catalyzed by T_4 RNA ligase were then determined for the activated donors A(5′)pp(5′)Gp and A(5′)pp(5′)Cp in the presence of a "good" acceptor (ApApA) and a "poor" acceptor (UpUpU). From the Lineweaver–Burk plot of Figure 3 the apparent K_m for A(5′)pp(5′)Gp in the presence of 0.5 mM ApApA was determined as 0.60 mM with an apparent V_{max} of 4700 nmol min^{-1} mg^{-1}. In the presence of 0.7 mM A(5′)pp(5′)Gp an apparent K_m for the acceptor ApApA of 0.18 mM and a V_{max} of 4300 nmol min^{-1} mg^{-1} were determined for the joining reaction (Figure 3). A(5′)pp(5′)Cp gave very similar kinetic parameters (Table I).

In attempting to measure the kinetic parameters for A(5′)pp(5′)Gp or A(5′)pp(5′)Cp in the presence of the acceptor UpUpU the initial velocity measurements indicated that substrate inhibition occurred particularly in the case of A(5′)pp(5′)Gp at concentrations of activated donor above 0.4

Table I: Kinetic Parameters for Phosphodiester Bond Formation by T_4 RNA Ligase Using Preadenylated Donors[a]

		kinetic parameters of the adenylated donor	
acceptor	adenylated donor	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})
ApApA	A(5′)pp(5′)Cp	0.80 ± 0.2	5000 ± 350
UpUpU	A(5′)pp(5′)Cp	0.80 ± 0.2	1250 ± 205
ApApA	A(5′)pp(5′)Gp	0.60 ± 0.2	4700 ± 400
UpUpU	A(5′)pp(5′)Gp	0.60 ± 0.2	2500 ± 210
ApApA	A(5′)pp-Bzl-NO_2	0.81 ± 0.2	60 ± 10
		kinetic parameters of the acceptor	
acceptor	adenylated donor	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})
ApApA	A(5′)pp(5′)Gp	0.18[b] ± 0.06	4300 ± 450

[a] A 0.1-mL reaction mixture contained 50 mM HEPES, pH 8.4, 3.3 mM dithiotreitol, 20 mM $MgCl_2$, 10 μg/mL bovine serum albumin, 32 μg/mL T_4 RNA ligase, 0.5 mM ApApA or UpUpU, and from 0.13 to 1.0 mM A(5′)pp(5′)Gp or A(5′)pp(5′)Cp. [b] Conditions as above but contained 0.7 mM A(5′)pp(5′)Gp and from 0.2 to 1.0 mM ApApA or UpUpU.

mM (Figure 3). However, by use of substrate concentrations of less than 0.4 mM an apparent K_m was measured which corresponded to that observed in the presence of ApApA (Table I). At substrate concentrations in excess of 0.4 mM the inverse of the initial velocity measured was plotted vs. the concentration of activated donor (Dixon & Webb, 1979). This allowed the calculation of a substrate inhibition constant (K_i^s) of 0.8 mM for A(5′)pp(5′)Gp in the presence of UpUpU. The kinetic parameters for UpUpU in the presence of 0.7 mM A(5′)pp(5′)Gp could not be reliably obtained as a result of the observed substrate inhibition.

T_4 RNA ligase has also been reported to accept P^2-substituted ADP derivatives as donors (England et al., 1977). An elegant method for blocking the 3′-terminal hydroxyl of a donor oligonucleotide using the photosensitive *o*-nitrobenzyl phosphate group has been previously described (Ohtsuka et al., 1979). In this case A(5′)pp-Bzl-NO_2 is prepared as an adenylated substrate for the enzyme. In order to compare the enzyme's ability to use adenylated derivatives of nonnucleotidic material, we determined the kinetic parameters for the reaction between ApApA and the adenylated *o*-nitrobenzyl phosphate derivative (Table I). The apparent K_m is nearly identical with those measured for A(5′)pp(5′)Gp and A(5′)pp(5′)Cp, but the velocity is some 80-fold slower.

While polymer elongation catalyzed by T_4 RNA ligase occurs in high yield with good substrates (purine-containing acceptors and pyrimidine-containing donors), the efficiency of phosphodiester bond formation decreases dramatically in the case of poor substrates (uridine-containing acceptors and guanosine-containing donors). On the basis of the kinetic analysis of the donor adenylation reaction as compared with the subsequent joining of the adenylated donor to suitable acceptor, it is clear that the enzyme exhibits more specificity in the former step. pGp is the poorest donor in the adenylation reaction. It has also been reported as the poorest donor of the four nucleoside 3′,5′-bisphosphates in the overall joining reaction when examined in the presence of a common acceptor (England & Uhlenbeck, 1978; Kikuchi et al., 1978; Romaniuk et al., 1982). We examined the possibility of increasing product yields in the overall joining reaction by simply increasing the substrate concentration of pGp to 10 mM in an attempt to enhance the rate of the donor adenylation step. By use of the acceptor oligoribonucleotide ApApA (0.5 mM) and a pGp concentration of 1.0 or 10 mM in the presence of ATP and 64 μg/ml T_4 RNA ligase at 37 °C, the reaction was analyzed at various times during a 3-h incubation as is illustrated in Figure 4. At all times analyzed the reaction mixture containing 10 mM pGp exhibited significantly more joined product than that observed with 1.0 mM pGp. The reaction using 1.0 mM pGp is 66% completed after a 3-h incubation at 37 °C. A second addition of enzyme can result in near quantitative formation of ApApApGp. If, however, the chemically activated donor A(5′)pp(5′)Gp at a concentration of 0.7 mM is used under the same reaction conditions, ApApApGp is quantitatively formed within 10 min (Figure 4). This observation is more dramatic in the case of UpUpU which is a poor substrate for the enzyme (England & Uhlenbeck, 1978; Ohtsuka et al., 1976; Romaniuk et al., 1982). In the presence of pGp only 8% of the acceptor is converted to product after a 3-h incubation (Figure 4). Under the same conditions the reaction of UpUpU in the presence of 0.7 mM A(5′)pp(5′)Gp is quantitative within 60 min. These observations are in agreement with those of Ohtsuka, who reported that chemically protected nucleoside pyrophosphate substrates exhibit less dependence on the acceptor oligoribonucleotide sequences (Ohtsuka et al., 1980a).

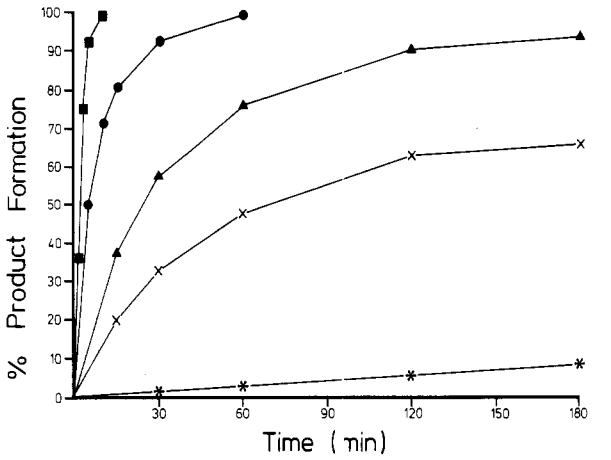

FIGURE 4: Joining reactions between nucleoside 3′,5′-bisphosphates or their adenylated derivatives with 0.5 mM ApApA or 0.5 mM UpUpU and 64 μg/mL T_4 RNA ligase in a 100 μL total volume. Other conditions as described in the legend to Figure 2. (*) UpUpU + 1.0 mM pGp + 3.3 mM ATP, (×) ApApA + 1.0 mM pGp + 3.3 mM ATP, (▲) ApApA + 10 mM pGp + 3.3 mM ATP, (●) UpUpU + 0.7 mM A(5′)pp(5′)Gp, and (■) ApApA + 0.7 mM A(5′)pp(5′)Gp.

We additionally examined the elongation of the pentamer CpApGpDpD corresponding to positions 13–17 of yeast tRNA specific for the amino acid phenylalanine. In order to prepare oligonucleotide fragments corresponding to the dihydrouridine loop of this tRNA, it was necessary to elongate the dihydrouridine-containing pentamer with guanosine. In the reaction of the acceptor substrate with pGp, ATP, and 64 μg/mL T_4 RNA ligase at either 37 °C for 6 h or 17 °C for 24 h no significant product was observed. However, the product CpApGpDpDpGp could be prepared in 50% yield by using a slight excess of A(5′)pp(5′)Gp at 17 °C for 24 h in the presence of 64 μg/mL T_4 RNA ligase (data not shown).

Discussion

While it has been previously reported that activation of the donor molecule in the T_4 RNA ligase reaction does not occur in the absence of the acceptor oligoribonucleotide (Uhlenbeck & Cameron, 1977), we have observed under similar conditions adenylation of pCp, pUp, pAp, and pGp. Formation of the activated donor occurs much faster in the case of ribonucleoside substrates than has been reported in the case of the

2′-deoxyribonucleoside bisphosphates measured in the presence of a 10-fold higher concentration of enzyme (Hinton et al., 1978). The enzyme appears to maintain significant activity with respect to adenylation of the bisphosphate substrates for a minimum of 6 h at 37 °C. This is in contrast to what is generally observed in the overall reaction where enzyme activity as observed from the production of joined oligomer appears to cease after roughly 60–180 min at 37 °C (Uhlenbeck & Cameron, 1977; L. W. McLaughlin, unpublished observations).

Accurate kinetic parameters for the donor adenylation reaction have been difficult to obtain since two steps are required, enzyme adenylation followed by formation of the nucleoside pyrophosphate derivative. It is possible that the rate of enzyme adenylation will vary with the pNp substrate and that enzyme adenylation may in some cases be rate limiting.

The relative extents of adenylation of a 1 mM solution of the nucleoside 3′,5′-bisphosphates after a 6-h incubation of 56% (pCp), 20% (pUp), 19% (pAp), and 6% (pGp) are in agreement with previous results which reported that phosphodiester bond formation between a common acceptor (ApApU) and the four nucleoside 3′,5′-bisphosphate donors occurred with yields of 74%, 23%, 12%, and 0% for pCp, pUp, pAp, and pGp, respectively, after 60 min at 37 °C with relatively low enzyme concentration (Romaniuk et al., 1982).

The rates at which the nucleoside 3′,5′-bisphosphates are activated in the absence of an acceptor substrate are clearly slower than what occurs in the presence of an acceptor oligoribonucleotide. It is possible that the acceptor molecule triggers a conformational change in the enzyme which enhances the rate of adenylation of the donor molecule by the enzyme as has been suggested previously (Sugino et al., 1977; Uhlenbeck & Cameron, 1977). On the other hand, this difference may result if the off rate of the adenylated donor from the enzyme is slower than the release of joined product by the enzyme as might be expected for an enzyme intermediate. We have been unable to measure the off rate for the activated donor or to successfully design a modified acceptor oligoribonucleotide which might exert the desired catalytic effect on the adenylation reaction without itself being consumed as substrate in the subsequent joining reaction.

Guanosine 3′,5′-bisphosphate is clearly the poorest of the four pNp substrates in the donor activation reaction. The inability of the enzyme to efficiently adenylate pGp and possibly longer donor molecules containing guanosine at the 5′-position may account for the difficulty in obtaining high yields of joined product when 5′-terminal guanosine donors are used (Ohtsuka et al., 1980b).

It is possible to increase the reaction yield of a synthesis involving pGp by using the bisphosphate donor at a higher concentration. With a good acceptor (ApApA) this has resulted in phosphodiester bond formation in over 90% yield after a 60-min incubation at 37 °C (Figure 4). Under similar conditions using 1.0 mM pGp the yield of product was 60%. The increased reaction yield may be a result of more efficient adenylation of guanosine 3′,5′-bisphosphate. This approach is, however, unlikely to be particularly useful for cases other than the single step elongation of a polymer using pGp.

The third mechanistic step of the ligase reaction, that of phosphodiester bond formation between an acceptor oligoribonucleotide and the preadenylated donor, occurs with high reaction yields. The apparent K_m values for the activated donors A(5′)pp(5′)Gp and A(5′)pp(5′)Cp appear to be essentially independent of the acceptor substrate. The similarity of the kinetic parameters determined for A(5′)pp(5′)Gp and A(5′)pp(5′)Cp suggests that in the joining reaction there is less specificity exhibited by the enzyme for the adenylated donor than has been observed for the overall joining reaction using pCp or pGp. This is in agreement with reports that indicate that the enzyme requires a nucleotide substrate for the ATP-dependent reaction (minimum substrate pNp) (Uhlenbeck & Gumport, 1982). However, in the subsequent joining reaction using an adenylated intermediate (ATP-independent reaction), a number of P_2-substituted ADP derivatives will act as donor substrates for the enzyme (England et al., 1977).

A comparison of the kinetic parameters for A(5′)pp(5′)Gp or A(5′)pp(5′)Cp with A(5′)pp-Bzl-NO_2 (Table I) indicates that while the apparent K_m values are very similar the velocity for the adenylated benzyl derivative is 80-fold slower than that of either the cytidine or guanosine derivative. This indicates that although the enzyme will use P_2-substituted ADP derivatives as substrates it does discriminate between nucleotidic and nonnucleotidic adenylated donors in the third step of the joining reaction.

The apparent K_m of the acceptor ApApA in the partial reaction involving the adenylated intermediate is much higher than observed for the circularization reaction involving $(pA)_n$ substrates (Kaufman et al., 1974). This may be related to the difference in substrate size, the fact that the present work deals only with the partial reaction or that in the previous report the substrate was both the acceptor and donor molecule.

Preadenylation of the nucleoside 3′,5′-bisphosphates significantly increases the rate at which the enzyme is able to catalyze phosphodiester bond formation. This is contrary to observations with DNA ligase where the rate of the partial reaction involving the DNA adenylate was observed to proceed slower than the overall reaction (Lehman, 1974). While this acceleration in the rate of phosphodiester bond formation appears to be true for both good and poor substrates, it is most noteworthy in the case of a poor acceptor substrate (UpUpU) and a poor donor substrate (pGp). Without preadenylation UpUpUpGp is formed in 8% yield after 3 h at 37 °C. With preadenylation the product is quantitatively formed within one hour (Figure 4). This is further illustrated by using the dihydrouridine-containing acceptor CpApGpDpD, which in the presence of the enzyme, pGp, and ATP did not undergo any significant elongation. However, by preadenylation of the donor a 50% conversion to product could be observed. This suggests that, in the case of poor reactions, product yields can be improved by using a preadenylated donor molecule.

In conclusion, the efficiency of the enzyme to catalyze the joining of a particular acceptor and donor molecule is greatly enhanced if the preadenylated donor is used. The joining of the adenylated donor and acceptor molecule exhibits less dependence on the sequence of the acceptor. It should therefore be possible to increase reaction yields and/or decrease the quantity of enzyme required for a particular polymer elongation by using the preadenylated form of the donor substrate.

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Registry No. A(5′)pp(5′)Cp, 67035-83-0; A(5′)pp(5′)Gp, 91811-22-2; A(5′)pp-Bzl-NO_2, 67030-28-8; ApApA, 917-44-2; RNA ligase, 37353-39-2.

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Nitrogenase Reactivity: Azide Reduction†

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ABSTRACT: We have examined the reduction of azide by the purified component proteins of nitrogenase (*Av*1 and *Av*2). One of the two species present in azide solutions, HN_3, was shown to be a potent substrate (K_m = 12 μM) which is reduced by six electrons to $N_2H_4 + NH_3$. HN_3 reduction does not yield any less highly reduced products, which implies the presence of tightly bound intermediates. HN_3 appears to be an effective inhibitor of H_2 evolution and to bind to a redox state of the enzyme more oxidized than that responsible for N_2 fixation or H_2 evolution. The other species present in solution, N_3^-, was shown to be the substrate reduced by two electrons to yield $N_2 + NH_3$. N_3^- is the only known anionic nitrogenase substrate, and its reduction is the only example of a nitrogenase reaction requiring inequivalent numbers of protons and electrons. Infinite [N_3^-] cannot eliminate H_2 evolution, and N_3^- may bind to and be reduced by both high and low redox states of the enzyme. Some of the N_2 formed appears to be further reduced by six electrons to two NH_3 in a reaction that is inhibited by D_2. The N_2 formed from N_3^- reduction, which is subsequently reduced to $2NH_3$, is not in equilibrium with N_2 in the gas phase. This suggests strongly that the N_2 must be formed at or near the N_2 reduction site.

Nitrogenase is composed of two separately purified proteins called the molybdenum-iron protein (MoFe protein) and the iron protein (Fe protein), whose physical properties have been reviewed recently (Orme-Johnson et al., 1977; Mortenson & Thorneley, 1979; Burgess, 1984). The MoFe protein contains the site of substrate reduction (Hageman & Burris, 1979; Shah et al., 1973) while the Fe protein is generally accepted as a specific electron donor for the MoFe protein (Hageman & Burris, 1978a; Ljones & Burris, 1978a,b). In addition to these two proteins, a source of reducing equivalents, MgATP, protons, and an anaerobic environment are required for all substrate reductions (Bulen & LeComte, 1966). Nitrogenase catalyzes not only the reduction of the physiological substrates dinitrogen and protons but also the reductions of the alternative substrates, nitrous oxide, acetylene, azide, cyanide, alkyl cyanides, alkyl isocyanides, hydrazine, cyclopropene, allene, and diazirine. Alternative substrates have often been studied as probes for the number and nature of sites of substrate interaction on nitrogenase and the types of intermediates that might be formed during N_2 reduction. This paper will describe recent studies on the reduction of azide by the purified component proteins of *Azotobacter vinelandii* nitrogenase.

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