T4 RNA ligase catalyzes the synthesis of dinucleoside polyphosphates

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T4 RNA ligase has been shown to synthesize nucleoside and dinucleoside 5'-polyphosphates by displacement of the AMP from the E-AMP complex with polyphosphates and nucleoside diphosphates and triphosphates. Displacement of the AMP by tripolyphosphate (P_3) was concentration dependent, as measured by SDS/PAGE. When the enzyme was incubated in the presence of 0.02 mM [α - ^{32}P] ATP, synthesis of labeled Ap_4A was observed: ATP was acting as both donor (K_m , μM) and acceptor (K_m , mM) of AMP from the enzyme. Whereas, as previously known, ATP or dATP (but not other nucleotides) were able to form the E-AMP complex, the specificity of a compound to be acceptor of AMP from the E-AMP complex was very broad, and with K_m values between 1 and 2 mM. In the presence of a low concentration (0.02 mM) of [α - ^{32}P] ATP (enough to form the E-AMP complex, but only marginally enough to form Ap_4A) and 4 mM of the indicated nucleotides or P_3 , the relative rate of synthesis of the following radioactive (di)nucleotides was observed: Ap_4X (from XTP, 100); Ap_4dG (from dGTP, 74); Ap_4G (from GTP, 49); Ap_4dC (from dCTP, 23); Ap_4C (from CTP, 9); Ap_3A (from ADP, 5); Ap_4ddA , (from ddATP, 1); p_4A (from P_3 , 200). The enzyme also synthesized efficiently Ap_3A in the presence of 1 mM ATP and 2 mM ADP. The following T4 RNA ligase donors were inhibitors of the synthesis of Ap_4G : $pCp > pAp > pA2'p$.

Keywords: RNA ligase; dinucleoside polyphosphates; Ap_3A ; RNA joining; firefly luciferase.

Dinucleoside polyphosphates (Np_nN , a family of 5',5'-linked dinucleotides) are molecules involved in metabolic regulation and cell signaling [1–3]. These compounds, mainly diadenosine polyphosphates (Ap_nA), have been implicated in a variety of intra and extracellular roles such as: regulation of purine nucleotide metabolism, alarmones acting in the heat shock and oxidative stresses, transition-state analogues of some kinases, control of cell division, neurotransmitters, vasoregulators,

inhibitors of ATP-regulated K^+ channels, etc. [1] (reviewed in [2,3]). Interest in dinucleoside polyphosphates has increased after the discovery that the putative human tumor suppressor gene *FHIT* (for fragile histidine triad) encodes for a typical dinucleoside 5',5'''- P^1, P^3 triphosphate hydrolase (EC3.6.1.29) [4], an enzyme that cleaves Ap_3A to AMP and ADP [5–8]. Crystal structure of the *fhit* protein showing its interaction with Ap_3A has been reported [9,10].

The hypothesis was put forward in 1990 that the enzymes ligases could be candidates for the synthesis of Np_nN [11]. Some ligases of the subclass EC 6.1 (as aminoacyl-tRNA synthetases) and of the subclass EC 6.2 (as acetyl-CoA synthetases and acyl-CoA synthetases) catalyze the synthesis of diadenosine tetraphosphate and other dinucleoside polyphosphates through the general reactions shown in Eqns (1) and (2) below [12–14]:

where X represents a free acid.

In addition, the oxidoreductase firefly luciferase (EC1.12.13.7) also catalyzes the synthesis of Ap_4A and other dinucleoside polyphosphates [15] using luciferin as cofactor and through reactions similar to 1 and 2. These enzymes liberate pyrophosphate and use the intermediate E-acyl-AMP as donor of AMP for the synthesis of dinucleoside polyphosphates. As reaction 1 is reversible, the synthesis of Ap_4A is greatly favoured in the presence of pyrophosphatase.

The ligases belonging to the subclass EC 6.5 catalyze the formation of phosphoric ester bonds through an intermediate E-AMP. Three enzymes are recognized in this group: EC6.5.1.1 or DNA ligase (AMP forming); EC6.5.1.2 or DNA ligase (AMP-forming, NMN-forming) and EC6.5.1.3 or RNA ligase. The synthesis of dinucleoside polyphosphates by T4 DNA ligase (EC6.5.1.1) has been very recently reported [16].

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Abbreviations: Ap_3A , adenosine(5')triphospho(5')adenosine or diadenosine triphosphate; Ap_3X , adenosine(5')triphospho(5')xanthosine; Ap_4A , adenosine(5')tetraphospho(5')adenosine; Ap_4dA , adenosine(5')tetraphospho(5')deoxyadenosine; Ap_4dC , adenosine(5')triphospho(5')deoxycytidine; Ap_4ddA , adenosine(5')tetraphospho(5')-2',3'-dideoxyadenosine; Ap_4dG , adenosine(5')tetraphospho(5')deoxyguanosine; Ap_4G , adenosine(5')tetraphospho(5')guanosine; Ap_4N , adenosine(5')tetraphospho(5')nucleoside; Ap_4X , adenosine(5')tetraphospho(5')xanthosine; Ap_5A , adenosine(5')pentaphospho(5')adenosine; ATP γ S, adenosine-5'- γ -thiotriphosphate; ddATP, 2',3'-dideoxyadenosine-5'-triphosphate; LH_2 , luciferin; Np_nN , nucleoside(5')polyphospho(5')nucleoside; P_3 , tripolyphosphate; P_4 , tetrapolyphosphate; P_{15} , linear-chain polyphosphate with an average chain length of 15 ± 3 p_4A , adenosine-5'-tetraphosphate; Xp_4X , xanthosine(5')tetraphospho(5')xanthosine.

Enzymes: acetyl-CoA synthetase (EC6.2.1.1); acyl-CoA synthetase (EC6.2.1.3); alkaline phosphatase (EC3.1.3.1); DNA ligase (AMP forming) (EC6.5.1.1); DNA ligase (AMP-forming, NMN-forming) (EC6.5.1.2); firefly luciferase (EC1.13.12.7); inorganic pyrophosphatase (EC3.6.1.1); RNA ligase (EC2.7.4.3).

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The mechanism of RNA ligases, similar to that of T4 DNA ligases [17], involves three reversible steps shown below in Eqns (3)–(5).

Depending on whether the 5'-P-RNA donor and the 3'-OH-RNA acceptor belong to the same or different RNA molecules the reaction will be intramolecular or intermolecular, respectively. In the first case T4 ligase promotes circularization of RNA. The enzyme can use either RNA or DNA as substrates [18–22].

Here we report for the first time the ability of T4 RNA ligase to catalyze the synthesis of adenosine 5'-polyphosphates and of a variety of dinucleoside polyphosphates.

MATERIALS AND METHODS

Materials

T4 RNA ligase was purchased from Boehringer Mannheim (1449478; lot numbers, 84064023-08 and 84064024-08). Electrophoresis on polyacrylamide gels, in the presence of SDS, of those preparations presented a unique band (when stained with Coomassie Blue) with an apparent molecular mass of 42 kDa. The specific activities were about 1800 U·mg⁻¹ protein. One unit (U) is the enzyme activity required to catalyze the formation of 1 nmol of [5'-³²P]rA_{12–18} into a phosphatase-resistant form in 30 min at 37 °C. Poly(A), sodium tripolyphosphate (5633) (P₃), hexaammonium tetrapolyphosphate (P₄) and sodium phosphate glass Type 15 (P₁₅) were from Sigma (Refs 5758 and 6003, respectively). The purity of the polyphosphates was checked by ion-exchange chromatography essentially as described in [23]. While P₃ and P₄ were more than 97% and 90% pure, respectively; P₁₅ was a mixture of at least 20 different (poly)phosphates [23]. Alkaline phosphatase (grade I) (EC 3.1.3.1), phosphodiesterase from *Crotalus durissus* (EC 3.1.4.1) and yeast inorganic pyrophosphatase (EC 3.6.1.1), were obtained from Boehringer Mannheim. pd(A)_{12–18} was from Pharmacia Biotech (Ref. 27-7975). [α-³²P]ATP, 3000 Ci·mmol⁻¹, was from Dupont NEN. TLC silica-gel fluorescent plates were from Merck. X-ray films were from Konica Corporation (Japan). Radioactively labeled nucleotides were quantified with the help of an InstantImager (Packard Instrument Co). HPLC was carried out in a Hewlett Packard chromatograph (model 1090), with a diode array detector, commanded by an HPLC ChemStation. The Hypersil ODS columns (2.1 × 100 mm) or (4.6 × 100 mm) were from Hewlett Packard. Ultrafiltration was performed with microconcentrators with exclusion limit membranes of 30 kDa from Vivascience.

Inorganic pyrophosphatase (used to hydrolyze the PP_i produced during the enzyme assays or the PP_i contaminating P₃) was a suspension in 3.2 M ammonium sulfate. As ammonium sulfate was an inhibitor of the reactions here described, pyrophosphatase was desalted by ultrafiltration before use.

T4 RNA ligase-AMP complex formation

The reaction mixture (0.02 mL) contained 50 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 5 mM MgCl₂, 5 μM (0.1 μCi) [α-³²P]ATP, 0.02 U of desalted pyrophosphatase and, when

indicated tripolyphosphate (P₃). The mixture was incubated for 15 min to remove the potential PP_i contaminating the commercial preparation of P₃. The formation of the E-AMP complex was initiated by addition of 2 U of T4 RNA ligase. After 15 min incubation, reactions were stopped with 6.5 μL of concentrated SDS sample buffer (0.25 M Tris/HCl (pH 6.8), 8% SDS, 40% glycerol, 240 mM dithiothreitol, 0.005% bromophenol blue). The mixtures were heated at 90 °C for 3 min and 6-μL aliquots loaded onto a 12% denaturing polyacrylamide gel. The gel was stained with Coomassie Blue, dried down and the labeled enzyme-adenylate complex detected by autoradiography.

Synthesis of (di)nucleoside polyphosphates by T4 RNA ligase

Unless otherwise indicated the reaction mixtures contained 50 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 5 mM MgCl₂, 1 U·mL⁻¹ of desalted pyrophosphatase, T4 RNA ligase, ATP and other nucleotides or polyphosphates as indicated. After incubation at 30 °C the reaction mixtures were analyzed by one of the following methods.

TLC. The reaction mixtures (0.02 mL) contained 0.02 mM [α-³²P]ATP (0.1 μCi). Aliquots (2 μL) of the reactions were taken, spotted on silica gel plates, along with standards, and developed in dioxane : ammonium hydroxide : water (6 : 1 : 4, or 6 : 1 : 6 by volume). Nucleotide spots were localized with a 253-nm wavelength light and the corresponding radioactivity measured by autoradiography and/or with an InstantImager.

HPLC. Aliquots (0.01 mL) of the reaction mixtures (usually in a volume of 0.05–0.1 mL) were transferred into 0.14 mL of water and kept at 95 °C for 1.5 min. After chilling, the mixtures were filtered and a 0.05-mL aliquot injected into a Hypersil ODS column (2.1 × 100 mm or 4.6 × 100 mm), as indicated. Elution was performed (for both types of columns) at a flow rate of 0.5 mL·min⁻¹ with a 20-min linear gradient (5–30 mM) of sodium phosphate (pH 7.5), in 20 mM tetrabutylammonium bromide/20% methanol (buffer A) followed, when pertinent, by a 10-min linear gradient (30–100 mM) of sodium phosphate (pH 7.5) in buffer A.

RESULTS AND DISCUSSION

Formation of the E-AMP complex and its reaction with P₃

Formation of a E-AMP complex was observed when T4 RNA ligase was incubated with [α-³²P]ATP. As expected, the complex migrated on SDS/PAGE in a position corresponding to a molecular mass of about 45 kDa [19] (Fig. 1a). In those experimental conditions (i.e. absence of PP_i because of the presence of pyrophosphatase), the AMP moiety of the complex reacted with P₃ in a concentration dependent manner (Fig. 1b).

Synthesis and characterization of Ap₄A catalyzed by T4 RNA ligase. Dependence on time and influence of pyrophosphatase.

The fate of AMP from the E-AMP complex was followed by incubating the enzyme with [α-³²P]ATP, in the absence or presence of pyrophosphatase. The reaction was followed by TLC (Fig. 2a,b). The radioactivity under the ATP spot was gradually displaced towards a new radioactive compound co-migrating with a standard of Ap₄A. In these experimental conditions the reaction was linear during 10 min (Fig. 2c). The synthesis of Ap₄A decreased with time of incubation, mainly

Fig. 1. Inhibition of T4 RNA ligase-adenylyl complex formation by tripolyphosphate (P₃). (a) The enzyme was incubated with [α -³²P]ATP in the absence or presence of increasing concentrations of P₃ as indicated, and subjected to SDS/PAGE as described in Materials and methods. The relevant portion of the autoradiogram is shown. The size (in kDa) of co-electrophoresed marker proteins are indicated by the arrow at the left. (b) The inhibition (%) of the adenylation of the enzyme by P₃ was quantified in an InstantImager.

because of the low ATP concentration in the mixture. The formation of the new spot, corresponding to Ap₄A was stimulated by desalted pyrophosphatase (Fig. 2a,b). The initial rates of synthesis of Ap₄A in the absence and presence of pyrophosphatase were 0.4 and 1.2 nmol·min⁻¹·mg⁻¹ protein, respectively. In the absence of pyrophosphatase, the calculated equilibrium constant of the reaction $2\text{ATP} \leftrightarrow \text{Ap}_4\text{A} + \text{PP}_i$ was 0.005.

The synthesis and characterization of Ap₄A by T4 RNA ligase was approached in a different reaction mixture (0.05 mL) containing 2 mM ATP, 10 U of ligase, and other conditions as described in Materials and methods. After overnight incubation, the reaction products were analyzed by HPLC. Most of the ATP was transformed into a new compound, Ap₄A. Minor peaks corresponding to AMP, ADP and to a presumptive Ap₃A were also observed (Fig. 3a). Ap₄A was characterized as follows. It co-eluted with an Ap₄A standard; treatment of the mixture with alkaline phosphatase (1 μ g for 2 h at 37 °C) degraded AMP and ADP to adenosine while keeping unaltered the chromatographic peaks corresponding to the presumptive Ap₄A and Ap₃A (Fig. 3b). After inactivation of the phosphatase, the reaction mixture was further treated with phosphodiesterase (0.4 μ g) for 10, 30, 60 and 120 min (Fig. 3c–f). The main products formed as a result of Ap₄A hydrolysis were ATP and AMP. ADP was the product of the hydrolysis of the presumptive Ap₃A by the phosphodiesterase.

K_m value for GTP in the synthesis of Ap₄G

The experiments shown in Figs 2 and 3 indicated that ATP behaves both as donor of AMP for the formation of E-AMP and acceptor of AMP for the formation of Ap₄A. The K_m value for ATP in the first step of the reaction (formation of the E-AMP complex) is around 10 μ M [18]. In our experience with other enzymes synthesizing Ap₄A [13–15], the second step (reaction 2)

is rather unspecific and any nucleoside triphosphate (NTP) can be acceptor of AMP, acting with a high K_m value. For these reasons, GTP was tested as acceptor of AMP, using labeled ATP as donor. The K_m value for GTP in the synthesis of Ap₄G was determined in the presence of fixed (0.02 mM) [α -³²P]ATP and variable (0.1 mM to 4 mM) GTP concentrations. In these conditions, the K_m value found for GTP was about 1.2 mM (result not shown), and the rate of synthesis (k_{cat}) of Ap₄G was 0.017 s⁻¹.

Effect of pH

The effect of pH on the rate of synthesis of a dinucleoside tetraphosphate (Ap₄G) was determined in reaction mixtures containing 0.02 mM [α -³²P]ATP (0.1 μ Ci), 4 mM GTP, and 0.8 U T4 RNA ligase and 50 mM (each) of the following buffers: Mes/KOH (for pH values of 5.5, 6.0, 6.4, 6.7); Hepes/KOH (for pH values of 6.7, 7.2, 7.6), Tris/HCl (for pH values of 7.4, 8). Aliquots were taken after 15 and 30 min incubation at 30 °C and analyzed by TLC. The reaction rate increased steadily from a pH value of 6.5 up to a maximum at pH 7.4. Beyond this point the enzyme activity decreased. At pH 8.0, the enzyme activity was around 70% of the maximum.

Substrate specificity

The following aspects of the substrate specificity of T4 RNA ligase were studied in the context of the synthesis of (di)nucleoside polyphosphates.

Nucleotides able to form an E-NMP complex (NMP = nucleoside 5'-monophosphate). This was approached indirectly according to the ability to form p₄N in the presence of 0.5 mM NTP + 3 mM P₃. The relative rates found were: ATP (100), dATP (6), CTP = GTP (< 1). These results are in

Fig. 2. Synthesis of diadenosine tetraphosphate by T4 RNA ligase: dependence on time and effect of pyrophosphatase. Two reaction mixtures were set up as described in Materials and methods, containing 0.02 mM [α -³²P]ATP in the absence (a) and presence (b) of desalted pyrophosphatase (PPase), 2 mM MgCl₂ and 6.6 U T4 RNA ligase. Aliquots were taken at the indicated times, subjected to TLC (a, b) and quantified in an InstantImager (c).

agreement with previous results indicating that ATP is the preferred substrate for the RNA joining reaction [18].

The ability of 3 mM P_3 or P_4 or P_{15} to act as adenylyl acceptors in the presence of 1 mM ATP was measured by HPLC. In a control mixture in the absence of polyphosphate,

transformation of ATP into Ap₄A was observed (Fig. 4A,a). In the presence of ATP + P_3 , synthesis of p₄A, Ap₄A and of very small amounts of Ap₅A took place (Fig. 4A,b), reflecting the following set of reactions: ATP + ATP $\rightarrow$ Ap₄A; ATP + P_3 $\rightarrow$ p₄A; and p₄A + ATP $\rightarrow$ Ap₅A. With P_4 as cofactor, only minor quantities of p₅A, along with Ap₄A, were obtained (Fig. 4A,c).

The commercial mixture of polyphosphates described as P_{15} was not a substrate for the reaction; in fact, these higher polyphosphates behaved as inhibitors of the reaction as no appreciable synthesis of Ap_4A was observed (Fig. 4A,d). The identity of the synthesized p_4A was investigated in an aliquot of the reaction mixture containing ATP + P_3 (Fig. 4B,a). The presumptive p_4A : (a) eluted with a standard of p_4A ; (b) was degraded by alkaline phosphatase to adenosine (and inosine, as a result of the adenosine deaminase contaminating the commercial preparations of alkaline phosphatase) (Fig. 4B,b); and (c) was hydrolyzed to AMP following treatment with snake venom phosphodiesterase (Fig. 4B,c).

Nucleotides as acceptors of AMP from the E-AMP complex. The K_m value described for ATP in the enzyme adenylation reaction was 12 μM [18]. The K_m value for ATP as acceptor of AMP was determined in the presence of 0.5, 1, 2, 4 and 6 mM ATP and fixed (1 mM) free Mg^{2+} . In these conditions the K_m found for ATP was 1.25 mM and the k_{cat} for Ap_4A synthesis was 0.055 s^{-1} . Hence, the synthesis of Ap_4N was approached in the presence of low concentration, 0.02 mM, of $[\alpha-^{32}P]$ ATP (enough to form Ap_4A), 0.8 U of T4 RNA ligase and 4 mM each of XTP, dGTP, GTP, dCTP, CTP, ADP, ddATP. After 15 min incubation, the products formed were separated by TLC and the radioactivity counted. The relative rates of synthesis measured for the corresponding dinucleotides were, respectively: Ap_4X (100); Ap_4dG (74); Ap_4G (49); Ap_4dC (23); Ap_4C (9); Ap_3A (5); Ap_4ddA (1). In the presence of 0.02 mM ATP and 4 mM P_3 p_4A was formed at a rate about double of that obtained for Ap_4X . The identity of these dinucleotides was assessed by their insensitivity to alkaline phosphatase, position in the TLC plate and, when possible, by their UV spectra (see below).

ATP γ S as a substrate of T4 RNA ligase. Because of its molecular characteristics, ATP γ S has been widely used as substrate of firefly luciferase using luciferin (LH2) as cofactor [15]. ATP γ S is a good adenylyl donor for formation of the E-LH₂-AMP complex (as it contains an intact α -phosphate within a P_3 chain) and a poor adenylyl acceptor (as it does not have an intact terminal PP_i). Because of these properties, this nucleotide has been used for the selective synthesis of Ap_4N by luciferase using ATP γ S and NTP (good AMP acceptors). Based on the above, ATP γ S was tested as substrate of T4 RNA ligase, using three reaction mixtures containing 2 mM ATP; ATP + XTP (2 mM each) or ATP γ S + XTP (2 mM each) (Fig. 5). In the first case (considered as a control) complete conversion of ATP into Ap_4A took place; small amounts of a presumptive Ap_3A were also observed (Fig. 5a). Synthesis of Ap_4A , Ap_4X and residual

Fig. 3. Synthesis and characterization of Ap_4A by T4 RNA ligase. (a) A reaction mixture (0.05 mL) containing 10 U of RNA ligase and 2 mM unlabeled ATP was incubated overnight and an aliquot (0.01 mL) subjected to HPLC as described in the text. Peaks corresponding to AMP, ADP and presumptive Ap_4A and Ap_3A were detected. (b) Another aliquot (0.04 mL) of the reaction mixture was treated with 1 μg of alkaline phosphatase for 2 h, heated in boiling water for 90 s, chromatographed and analyzed by HPLC. (using a Hypersil ODS column, 2.1 $\times$ 100 mm). The peaks corresponding to AMP and ADP were transformed into adenosine while the presumptive Ap_4A and Ap_3A remain unchanged. The remaining mixture (0.03 mL) was treated with snake venom phosphodiesterase (0.4 μg) for 10 (c), 30 (d), 60 (e) and 120 (f) min and analyzed by HPLC in the same conditions as above. AP, alkaline phosphatase; PDE, snake venom phosphodiesterase. The numbers on top of the peaks correspond to the following compounds: (1) AMP; (2) ADP; (3) Ap_3A ; (4) Ap_4A ; (5) Ado; (6) ATP.

Fig. 4. Polyphosphates as substrates of T4 RNA ligase. (A) The enzyme was incubated in the presence of 1 mM ATP, desalted pyrophosphatase and in the absence (a) or presence of 3 mM each of P₃ (b); P₄ (c) or P₁₅ (d). After overnight incubation at room temperature, the mixtures were analyzed by HPLC (using a Hypersil ODS column, 2.1 × 100 mm). (B) Two aliquots of the reaction mixture (a) were treated either with alkaline phosphatase (5 µg, 2 h, 37 °C) (b) or with snake venom phosphodiesterase (1 µg, 2 h, 37 °C) (c). All three samples were analyzed by HPLC (using a Hypersil ODS column, 4.6 × 100 mm). AP alkaline phosphatase; PDE phosphodiesterase. The numbers on top of the peaks correspond to the following compounds: (1) ATP; (2) Ap₄A; (3) p₄A; (4) Ap₅A; (5) p₅A; (6) adenosine; (7) inosine (8) AMP.

amounts of Ap₃A and Ap₃X were observed in the presence of ATP and XTP (Fig. 5c); the results were as expected, i.e. once the E-AMP complex was formed, the adenylyl residue could be transferred to either ATP (with formation of Ap₄A) or to XTP (with formation of Ap₄X). Pyrophosphatase can hydrolyze inorganic thiopyrophosphate (generated from ATPγS) to Pi + thiophosphate [24] thus preventing its reaction with the E-AMP complex. In the mixture containing ATPγS and XTP only a small amount of Ap₄X was synthesized (Fig. 5b), reflecting two facts: (a) XTP was not donor for the formation of an hypothetical E-XMP, as formation of Xp₄X would be expected in that case; (b) ATPγS was a poor donor for the formation of E-AMP.

The nature of the synthesized products was analyzed in several ways: (a) by their chromatographic position – in our experimental conditions, xanthine derivatives are retarded with respect to adenine derivatives of equal structure and number of phosphates; (b) by treatment with alkaline phosphatase of the mixture containing ATP and XTP – only peaks corresponding to Ap₄X, Ap₄A and to presumptive Ap₃X remained (Fig. 5d); and (c) by UV absorption spectra of Ap₄A (or Ap₃A) compared with that of Ap₄X (or Ap₃X) or XTP (Fig. 5e).

The relative amount of Ap₄X synthesized in the presence of (ATP + XTP) or (ATPγS + XTP) was 100 and 10, respectively.

The potential of ATP γ S to adenylylate RNA ligase was further assayed in the experiment described below.

Synthesis of diadenosine triphosphate catalyzed by T4 RNA ligase

A clue to the synthesis of Ap₃A by T4 RNA ligase was obtained in previous assays (Figs 3b and 5a). However, due to the recent interest of diadenosine triphosphate as substrate of a potential tumor suppressor gene (the *FHIT* gene [4–7]), the synthesis of Ap₃A was further assessed by HPLC in reaction mixtures containing 1 mM ATP + 2 mM ADP (Fig. 6a) or 2 mM ATP γ S + 2 mM ADP (Fig. 6b). After an overnight incubation, the mixtures were treated with alkaline phosphatase (Fig. 6c,d), and the remaining peaks corresponded to Ap₃A and Ap₄A. The nature of the presumptive Ap₃A was further confirmed by treatment with dinucleoside triphosphatase (purified from rat liver [6]), which gave ADP and AMP as products of hydrolysis of Ap₃A (results not shown).

In the presence of 1 mM ATP + 2 mM ADP (Fig. 6a), the relative amounts of Ap₄A and Ap₃A formed were 100 and 130, respectively. As above, ATP γ S was not good donor of AMP for the synthesis of Ap₃A from ADP: the Ap₃A synthesized in the presence of ATP γ S + ADP was only 25% of that formed in the presence of ATP + ADP.

Inhibition of the synthesis of Ap₄G by T4 RNA ligase donors

In the terminology at use for T4 RNA ligase [19,25], the 5'-phosphoryl and the 3'-OH terminated portions of RNA (or similar molecules) are called the RNA *donor* and the RNA *acceptor*, respectively (shown in *italics*). Three active sites are considered in the enzyme: the ATP site, the *donor* site and the *acceptor* site. Although the term *donor* refers more properly to the potentiality of being RNA *donor*, the 5'-P end of RNA is firstly acting as AMP acceptor from the E-AMP complex. Only after formation of E-AppRNA takes place, the RNA behaves as *donor*. In a strict sense, the term *acceptor* could be applied equally well to the modified 5'-P end of RNA, as this end can also be viewed as accepting the 3'-OH terminal RNA. It seems to us curious that this terminology has not been developed for the very similar reactions catalyzed by T4 DNA ligase. In the context of this work, it seems more appropriate to name the three reactions catalyzed by T4 RNA ligase as: enzyme adenylylation, RNA adenylylation and RNA joining.

The substrate specificity of the ATP binding site can be directly determined [18] and this work. The substrate specificity of the 5'-P-RNA *donor* in the RNA adenylylation reaction [4] has been indirectly determined by studying the overall RNA joining reaction [19]. A ribonucleoside 3',5'-bisphosphate (pCp > pAp > pA2'p) was much better *donor* than a nucleoside 5'-monophosphate (pA or pC). Related to the mechanism of reaction of this enzyme, the synthesis of p₄A (from ATP and P₃) or Ap₄N (from ATP and NTP) can be interpreted in two ways.

Fig. 5. ATP γ S as substrate of T4 RNA ligase. Among other components (see Materials and methods), the reaction mixtures contained 10 U RNA ligase and: (a) 2 mM ATP; (b) 2 mM ATP γ S + 2 mM XTP; (c) 2 mM ATP + 2 mM XTP. After an overnight incubation, mixture (c) was further treated with 1 μ g alkaline phosphatase for 2 h at 37 °C (d). Analysis was performed by HPLC. UV spectra corresponding to Ap₄A (4), or Ap₄X (8) or XTP (7) are indicated in part (e) of the Fig. The spectra of the presumptive Ap₃A; (3) and Ap₃X (9) were identical to the spectra of Ap₄A (4) and Ap₄X (8), respectively. The numbers on top of the peaks correspond to the following compounds: (1) AMP; (2) ADP; (3) Ap₃A; (4) Ap₄A; (5) XDP; (6) ATP γ S; (7) XTP; (8) Ap₄X; (9) Ap₃X; (10) Ado + Ino.

Fig. 6. Synthesis of Ap₃A catalyzed by T4 RNA ligase. Among other components (see Materials and methods), the reaction mixtures contained 10 U RNA ligase and: (a) 1 mM ATP + 2 mM (ADP); (b) 2 mM ATP γ S + 2 mM ADP. After overnight incubation, aliquots of the mixtures were treated with 1 μ g of alkaline phosphatase (c and d, respectively), and analyzed by HPLC. The numbers on top of the peaks correspond to the following compounds: (1) AMP; (2) ADP; (3) Ap₃A; (4) Ap₄A; (5) ATP γ S; (6) Ado + Ino.

Firstly, the formation of these compounds simply implies reversal of the enzyme adenylation reaction (reaction 3), i.e. the E-AMP complex transfers the AMP moiety to P₂, P₃, p₃A, p₃G, p₂A, etc., with formation of ATP, p₄A, Ap₄A, Ap₄G, Ap₃A, etc., respectively, without apparently any need of the *donor* substrate or *donor* site of the enzyme. Secondly, the same reactions could take place after location of P₃ (or NTP) in the *donor* site of the enzyme, and are adenylated there by the E-AMP complex. The substrate specificity of the *donor* site, as studied in the RNA joining reaction does not support this possibility [19].

Considering either of these two alternatives, a true *donor* of the enzyme, susceptible to being adenylated, could be expected to be an inhibitor of the synthesis of Ap₄A. Variable concentrations (0.01–0.25 mM) of RNA *donors* (pCp, pAp, pA2'p) were tested as inhibitors of the synthesis of Ap₄A at a fixed (0.02 mM) [α -³²P]ATP concentration (Fig. 7). The reaction mixtures were analyzed by TLC. Under our experimental conditions (see Materials and methods), Ap₄A and the presumably synthesized Ap₂Cp, Ap₂Ap, and Ap₂A2'p migrated in the same position (results not shown). Treatment with alkaline

phosphatase before separation by TLC of the products formed, allowed the separation of Ap₄A from Ap₂C and Ap₂A. The results obtained with pAp as inhibitor of the synthesis of Ap₄A are shown in Fig. 7a. Similar experimental evidence was obtained with pCp and pA2'p (results not shown). From these experiments, the inhibition of the synthesis of Ap₄A by the three RNA *donors* tested was calculated (Fig. 7b). The best inhibitor of the synthesis of Ap₄A was pCp followed by pAp and pA2'p, in good correlation with their effectiveness as substrate *donors* of T4 RNA ligase [19].

In a similar experiment, a fixed concentration (0.02 mM) of pCp, pAp, pA2'p was added to reaction mixtures containing 0.02 mM [α -³²P]ATP. In these conditions, the initial rates of synthesis of Ap₂Cp, Ap₂Ap and Ap₂A2'p were 12.5, 3.5 and 0.15 nmol·min⁻¹·mg⁻¹ protein, respectively, whereas the initial rate of synthesis of Ap₄A was 1.06 (with pCp and pAp) and 1.2 nmol·min⁻¹·mg⁻¹ (with pA2'p). In the absence of added nucleoside bisphosphate, the rate of synthesis of Ap₄A from ATP was 1.25 nmol·min⁻¹·mg⁻¹. Altogether, these results are in agreement with those presented in Fig. 7. On the other side, dephosphorylated poly(A) (2, 6, and 20 μ g) did not have an

Fig. 7. Inhibition of the synthesis of Ap₄A by T4 RNA ligase donors. The reaction mixtures contained a fixed amount (0.02 mM) of [α -³²P]ATP, other components as indicated in Materials and methods, 2 U T4 RNA ligase and variable concentrations of pAp, pA₂'p or pCp. After 60 min incubation, the reaction mixtures were treated with 1 μg alkaline phosphatase for 1 h at 37 °C, subjected to TLC and the radioactivity corresponding to Ap₄A, and to Ap₂A, or Ap₂C quantified in an InstantImager. (a) Autoradiography of the TLC plate of the experiment performed with pAp as inhibitor; (b) relative inhibition of the synthesis of Ap₄A by pAp, pA₂'p or pCp.

appreciable effect on the synthesis of Ap₄A analyzed in the same experimental conditions as above.

Conclusions

As shown in this paper, T4 RNA ligase catalyzes the transfer of AMP from the E-AMP complex to diverse compounds, generating the products indicated between parentheses: ATP (Ap₄A); NTP (Ap₄N), P₃ or P₄ (p₄A or p₅A); ADP (Ap₃A). In our view these reactions can be interpreted as a reversion of the

first step of reaction [3], i.e. $\text{ATP} + \text{E} \leftrightarrow \text{E-AMP} + \text{PP}_i$, where diverse polyphosphate derivatives can replace PP_i. The above reaction is favoured when the generated PP_i is removed by pyrophosphatase, and it does not require the presence of RNA donors or acceptors. According to reactions (3 and 4), RNA donors could be expected to be inhibitors of the reaction, as NTP and RNA compete for the same E-AMP complex. That was the case when the synthesis of Ap₄A was tested in the presence of the known RNA donors, pCp, pAp and pA₂'p. Their degree of inhibition of that synthesis was similar to their effectiveness described as RNA donors. By the contrary, an RNA acceptor, as dephosphorylated poly(A), does not compete with NTP for the E-AMP complex (compare Eqns 3 and 5) and would be expected not to be inhibitory of the synthesis of dinucleoside polyphosphates. The above opens perspectives referring to the potential use of (tri)polyphosphate derivatives as inhibitors of T4 RNA ligase.

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