T4 DNA ligase synthesizes dinucleoside polyphosphates

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Abstract T4 DNA ligase (EC 6.5.1.1), one of the most widely used enzymes in genetic engineering, transfers AMP from the E-AMP complex to tripolyphosphate, ADP, ATP, GTP or dATP producing p_4A, Ap_3A, Ap_4A, Ap_4G and Ap_4dA, respectively. Nicked DNA competes very effectively with GTP for the synthesis of Ap_4G and, conversely, tripolyphosphate (or GTP) inhibits the ligation of DNA by the ligase. As T4 DNA ligase has similar requirements for ATP as the mammalian DNA ligase(s), the latter enzyme(s) could also synthesize dinucleoside polyphosphates. The present report may be related to the recent finding that human Fhit (fragile histidine triad) protein, encoded by the *FHIT* putative tumor suppressor gene, is a typical dinucleoside 5′,5″-P^1,P^3-triphosphate (Ap_3A) hydrolase (EC 3.6.1.29).

Key words: Ap_3A; Ap_4A; p_4A; *FHIT* gene; T4 DNA ligase; Dinucleoside polyphosphate

1. Introduction

Dinucleoside polyphosphates (Np_nN) are compounds of increasing interest in metabolic regulation and in cell signalling [1–3]. Ap_4A is present at (sub)micromolar concentrations in all the prokaryotic and eukaryotic tissues examined [4]. Ap_4A and other Np_nN may participate in the regulation of purine nucleotides [5,6], in cellular response to stress [2], in the control of cell proliferation [7], in the interferon action [8], as transition state analogues of some kinases [9], etc. Ap_4A and other dinucleoside polyphosphates are present in chromaffin granules of bovine adrenal medulla [10–12], in synaptic terminals [13] and in human blood platelets [14]. After an appropriate stimulus they are released from these storage sites to the blood and through their interaction with some still not well defined purine receptors [15–19] they may modulate a variety of processes such as vascular tone [20], platelet aggregation [20,21], neurotransmission [22], cell proliferation [3,7,23], etc.

The interest in dinucleoside polyphosphates was rekindled after the discovery that the fragile histidine triad (*FHIT*), a putative tumor suppressor gene [24], encodes a typical dinucleoside triphosphate hydrolase (EC 3.6.1.29) [25], an enzyme that cleaves Ap_3A to AMP and ADP [26,27]. The crystal structure of the Fhit protein showing its interaction with Ap_3A has been reported [28,29].

The intracellular level of dinucleoside polyphosphates results from their rate of synthesis and degradation. A variety of enzymes have been described in mammals, plants, lower eukaryotes and prokaryotes able to cleave specifically dinucleoside polyphosphates [30]. There are also unspecific phosphodiesterases present in the outer aspect of the membranes of most mammalian cells examined that hydrolyze Np_nN to the corresponding nucleoside 5′-monophosphates [30–32]. It was believed, since 1966, that aminoacyl-tRNA synthetases were the enzymes responsible for the synthesis of Ap_4A in vivo [33]. In 1990 the hypothesis was put forward that those ligases which catalyze the transfer of a nucleotidyl moiety, via nucleotidyl containing intermediates and releasing PP_i, may catalyze the synthesis of Np_nN [34]. These ligases usually catalyze, in a first step, the formation of an E-X-AMP intermediate with liberation of PP_i. In a second step, the acyl residue (X) of the complex is joined to an alcohol (Y) giving rise to X-Y. In the presence of pyrophosphatase and in the absence of Y, the AMP moiety of the E-X-AMP complex can be transferred to a nucleoside triphosphate (NTP) generating Ap_4N. Firefly luciferase (although classified as an oxygenase, EC 1.13.12.7) catalyzes very effectively the synthesis of nucleoside 5′-polyphosphates (p_nA, $n=3$–20), diadenosine polyphosphates (Ap_nA, $n=4$–16), and a variety of dinucleoside tetraphosphates (Ap_4N, N = any nucleoside) using luciferin, ATP and polyphosphates or NTP as cofactors [35]. Yeast acetyl-CoA synthetase [36] and *Pseudomonas fragi* acyl-CoA synthetase [37] catalyze also, to a lesser extent, the synthesis of (di)nucleoside polyphosphates.

The synthesis of (di)nucleoside polyphosphates by T4 DNA ligase is here described for the first time.

2. Materials and methods

2.1. Materials

T4 DNA ligase was purchased from Pharmacia (ref. 27-0870; lot 7100870041). λ DNA/*Hin*dIII fragments were from Gibco BRL. Sodium tripoly- or trimetaphosphates were from Sigma. Inorganic pyrophosphatase (EC 3.6.1.1) and alkaline phosphatase (grade I) (EC 3.1.3.1) were obtained from Boehringer Mannheim. Dinucleoside tetraphosphatase (EC 3.6.1.17) was purified from rat liver as previously described [38]. Thin layer chromatographic (TLC) silica gel fluorescent plates were from Merck. For autoradiography X-ray films from Konica Corporation (Japan) were used. The amount of labelled nucleotides was quantified with 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.

2.2. T4 DNA ligase-AMP complex formation

The reaction mixture (0.02 ml) contained 50 mM HEPES-KOH pH 6.7, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.01 mM (0.1 μCi) [α-^{32}P]ATP, 0.02 U of desalted pyrophosphatase and, when indicated,

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Abbreviations: Ap_3A, diadenosine triphosphate or adenosine(5′)-triphospho(5′)adenosine; Ap_4A, diadenosine tetraphosphate or adenosine(5′)tetraphospho(5′)adenosine; Ap_4dA adenosine(5′)tetraphospho(5′)2′-deoxyadenosine; Ap_4G, adenosine(5′)tetraphospho(5′)guanosine; Ap_4N, adenosine(5′)tetraphospho(5′)nucleoside; Np_nN, dinucleoside polyphosphates; meta P_3, trimetaphosphate; P_3, tripolyphosphate; p_4A, adenosine 5′-tetraphosphate

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tripolyphosphate (P_3) or GTP. The mixture was incubated for 15 min to remove the potential PP_i contaminating the commercial preparations of P_3 or GTP. The formation of the E-AMP complex was initiated by addition of 0.49 μg (3438 Weiss units/mg) of T4 DNA 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 15 μl aliquots loaded onto a 12% denaturing polyacrylamide gel. The gel was stained with Coomassie blue, dried down and the labelled enzyme-adenylate complex detected by autoradiography.

2.3. Synthesis and characterization of (di)nucleoside polyphosphates

The reaction mixtures (Figs. 2–4) were analyzed by TLC or HPLC. In the first case, aliquots (2 μl) of the reactions were taken, spotted on a TLC silica gel plate, along with standards of Ap_4G and p_4A (Fig. 2), or Ap_3A, Ap_4A and Ap_4dA (Fig. 3), and developed in dioxane:ammonium hydroxide:water (6:1:6, by volume) (Figs. 2 and 4) or in dioxane:ammonium hydroxide:water (6:1:4, by volume) (Fig. 3). Nucleotide spots were localized with a 253 nm wavelength light and the corresponding radioactivity measured by autoradiography and/or with an InstantImager. When analyzed by HPLC, aliquots (0.01 ml) of the reaction were transferred into 0.14 ml hot 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. Elution was performed at a flow rate of 0.5 ml/min 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.

2.4. DNA ligation

The reaction mixture (0.02 ml) contained 50 mM HEPES-KOH pH 7.2, 1 mM dithiothreitol, 0.02 mM ATP, 0.02 U desalted pyrophosphatase, 0.25 μg of λ DNA/*Hin*dIII fragments, P_3 or trimetaphosphate (meta-P_3) as indicated and 5 mM $MgCl_2$ in excess over that of tri(meta)phosphate. Reaction mixtures were incubated at 30°C for 30 min (to remove contaminant PP_i); thereafter 0.03 μg of T4 DNA ligase was added and, at the indicated times, 5 μl aliquots of the reaction mixtures were taken, added to 1 μl of a solution containing 0.25% bromophenol blue, 30% glycerol, 125 mM EDTA, heated for 10 min at 65°C and loaded onto wells of an 0.8% agarose gel. After electrophoresis ligation products were visualized by ethidium bromide staining.

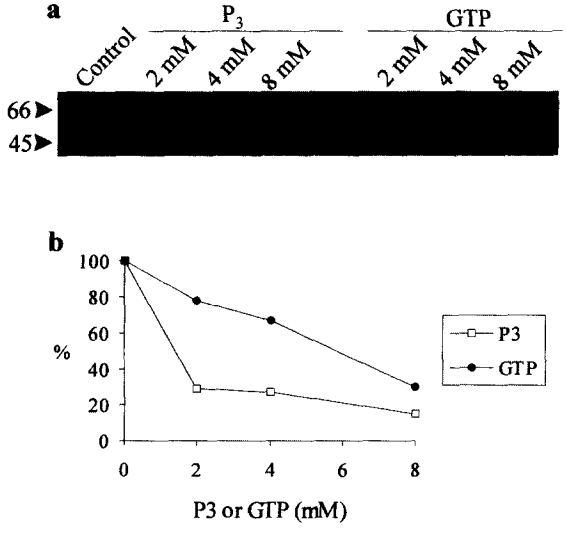

Fig. 1. Inhibition of T4 DNA ligase-adenylate complex formation by tripolyphosphate (P_3) or GTP. a: The relevant portion of the autoradiogram is shown. The sizes (in kDa) of coelectrophoresed marker proteins are indicated by the arrows on the left. b: The inhibition (%) of adenylation of the enzyme by P_3 or GTP was quantified in an InstantImager.

3. Results and discussion

3.1. Formation of the E-AMP complex and its reaction with tripolyphosphate (P_3) or GTP

T4 DNA ligase, one of the most widely used enzymes in genetic engineering, belongs to the group of ATP-dependent ligases, in which the mammalian DNA ligases are included [39,40]. It catalyzes the formation of phosphodiester bonds between neighboring 3′-hydroxyl and 5′-phosphate ends in double-stranded DNA (reactions 1–3):

$$E + ATP \rightarrow E\text{-}AMP + PP_i \quad (1)$$

$$E\text{-}AMP + DNA\text{-}5'\text{-}P \rightarrow E{\cdot}DNA\text{-}5'\text{-}PP\text{-}'5A \quad (2)$$

$$E{\cdot}DNA\text{-}5'\text{-}PP\text{-}'5A + HO\text{-}3'\text{-}DNA \rightarrow E + DNA\text{-}5'\text{-}P\text{-}3'\text{-}DNA + AMP \quad (3)$$

When T4-DNA ligase was incubated with [α-^{32}P]ATP formation of the complex E-AMP was observed (Fig. 1a), as expected, in a position corresponding to a molecular mass of about 57 kDa [40]. In the presence of pyrophosphatase, the AMP moiety of the complex was displaced by P_3 or by GTP, in a concentration dependent manner (Fig. 1b).

3.2. Synthesis of p_4A and Ap_4G catalyzed by T4 DNA ligase

The fate of AMP from the E-AMP complex was followed by incubating the enzyme in the absence of DNA and in the presence of [α-^{32}P]ATP and P_3 or GTP. The reaction was followed by TLC (Fig. 2a,b). The radioactivity under the ATP spot was gradually displaced towards new radioactive compounds corresponding, presumably, to p_4A and Ap_4G (Fig. 2). The nature of the newly synthesized radioactive products was assessed as p_4A and Ap_4G by the following criteria: comigration (TLC) or coelution (HPLC) with standards; treatment with alkaline phosphatase; treatment of the radioactive [adenylate-^{32}P]ApppppG with purified rat liver dinucleoside tetraphosphatase [30,38] which gave radioactive chromatographic spots corresponding to [α-^{32}P]AMP and [α-^{32}P]ATP (Fig. 2c). The synthesis of p_4A was further analyzed by HPLC. It eluted in the same position as a standard of p_4A (Fig. 2d). Treatment of the synthesized p_4A with alkaline phosphatase gave chromatographic peaks corresponding to ATP, ADP, AMP and adenosine (Fig. 2d). The synthesis of p_4A and Ap_4G by T4 DNA ligase was unequivocally demonstrated.

3.3. Synthesis of Ap_3A, Ap_4A and Ap_4dA catalyzed by T4 DNA ligase

The K_m values for ATP in the first step of the reaction (formation of the E-AMP complex) is in the order of μM [41]. The apparent K_m values determined for P_3 and GTP in the second step of the reaction (formation of p_4A and Ap_4G, respectively) are in the order of mM (results not shown). These results were reminiscent of those obtained with firefly luciferase [35] and other ligases [36,37] in which the K_m values for the AMP acceptor substrate (P_3, ATP, GTP, etc.) were in the mM range. In spite of these limitations, DNA ligase catalyzes the synthesis of Ap_4A, using 20 μM ATP as substrate (Fig. 3, lanes 2–4). T4 DNA ligase was also able to transfer

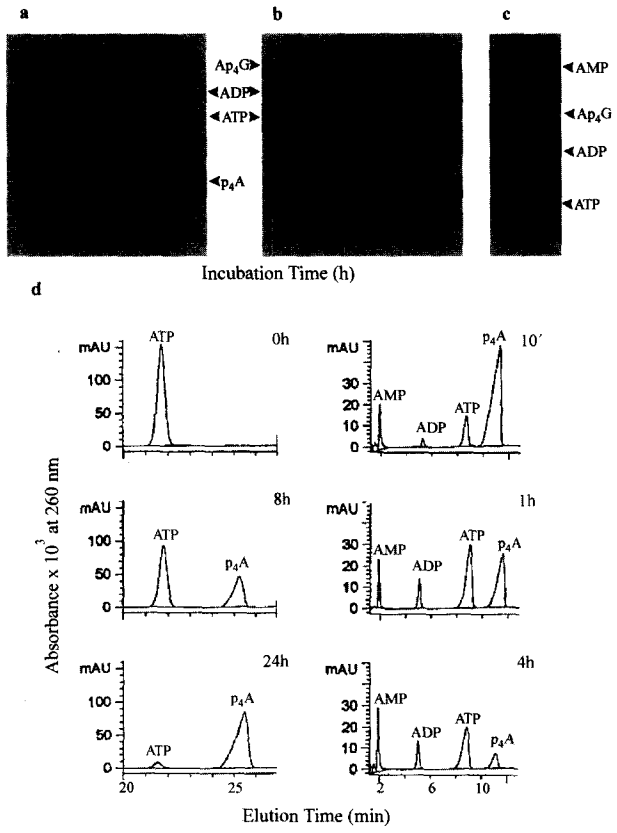

Fig. 2. Synthesis of adenosine tetraphosphate (p_4A) and adenosine-(5′)tetraphospho(5′)guanosine (Ap_4G) catalyzed by T4 DNA ligase. a, b: The reaction mixtures (0.03 ml) contained 50 mM HEPES-KOH (pH 7.2), 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.02 mM (0.2 μCi) [α-^{32}P]ATP, 0.03 U desalted pyrophosphatase, 0.74 μg T4 DNA ligase and 1 mM P_3 (a) or 1 mM GTP (b). Aliquots of the reactions were taken, at the indicated times, and analyzed by TLC (a, b) (see Section 2). c: Characterization of the synthesized Ap_4G. The remainder of the reaction mixture described in (b) was treated with 0.4 mU of dinucleoside tetraphosphatase and, after 3 h incubation at 37°C, analyzed by TLC. d: Synthesis and characterization of p_4A followed by HPLC. The reaction mixture (0.1 ml) for the synthesis contained 50 mM HEPES-KOH (pH 7.2), 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.03 U desalted pyrophosphatase, 0.74 μg T4 DNA ligase, 1 mM ATP, and 3 mM P_3. Aliquots were taken at the indicated times and analyzed using a 4.6×100 mm Hypersil ODS column (panel at the left). The remainder of the mixture (0.06 ml) was treated with 1 U alkaline phosphatase and the products of the reaction analyzed using a 2.1×100 mm Hypersil ODS column (panel at the right).

the AMP moiety of the E-AMP complex to ADP or dATP yielding Ap_3A (Fig. 3, lanes 5–7) and Ap_4dA (Fig. 3, lanes 8–10), as characterized by coelution with markers (Fig. 3).

3.4. P_3 inhibits the ligation of DNA by T4 DNA ligase

From Eqs. 1–3 it appears that P_3 or GTP could compete with DNA for the same E-AMP complex. In fact, DNA competes very effectively with GTP for the synthesis of Ap_4G (Fig. 4) and conversely P_3 (or GTP, not shown) inhibited the ligation of λ DNA/*Hin*dIII fragments by the ligase (Fig. 5).

3.5. Concluding remarks

T4 DNA ligase belongs to the subclass EC 6.5 and, contrary to other ligases of subclasses EC 6.1 and EC 6.2, is both acceptor and donor of AMP without any need for an E-X-

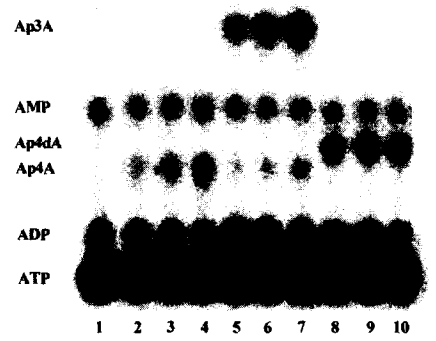

Fig. 3. Synthesis of Ap_4A, Ap_3A and Ap_4dA catalyzed by T4 DNA ligase. The reaction mixture (0.02 ml) contained 50 mM MES-KOH pH 6.7, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.02 mM (0.1 μCi) [α-^{32}P]ATP, 0.02 U desalted pyrophosphatase and 0.49 μg of T4 DNA ligase. Synthesis of Ap_4A: lanes 1–4, reaction times 0, 1, 2 and 4 h, respectively. Synthesis of Ap_3A or Ap_4dA: the above mixture was supplemented with 4 mM ADP (lanes 5–7) or 4 mM dATP (lanes 8–10); aliquots were taken after 1, 2 and 4 h of incubation, respectively, and analyzed by TLC as indicated in Section 2.

AMP intermediate [35–37]. As T4 DNA ligase has similar requirements for ATP as the mammalian DNA ligases [39,40], the latter enzymes could also synthesize Np_nN. This opens perspectives relating these compounds to DNA replication. In this regard, dinucleoside tetraphosphatase has been reported in the nuclei of tomato cells [42], but not in mammals; there are reports relating Ap_4A or Ap_3A to cell division [1–3,7,23]; the capacity of synthesis of Ap_3A (and other dinucleoside polyphosphates) by a DNA ligase and the role given to the dinucleoside triphosphatase as a tumor suppressor activity could be interrelated; finally, the development of new antitumor drugs, particularly some (tri)polyphosphates derivatives, inhibitors of DNA ligases, could be envisaged.

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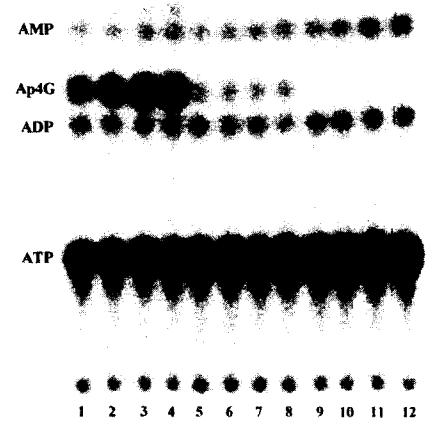

Fig. 4. Synthesis of Ap_4G catalyzed by T4 DNA ligase. Effect of DNA. The reaction mixture (0.02 ml) contained 50 mM MES-KOH pH 6.7, 1 mM dithiothreitol, 2 mM $MgCl_2$, 0.02 mM (0.1 μCi) [α-^{32}P]ATP, 1 mM GTP, 0.02 U desalted pyrophosphatase, 0.49 μg T4 DNA ligase, and variable amounts of λ DNA/*Hin*dIII fragments: 0 μg (lanes 1–4); 0.5 μg (lanes 5–8); 5 μg (lanes 9–12). Aliquots of the reaction were taken after 0.5, 1, 2 and 3 h of incubation, respectively, spotted on TLC and analyzed as described in Section 2.

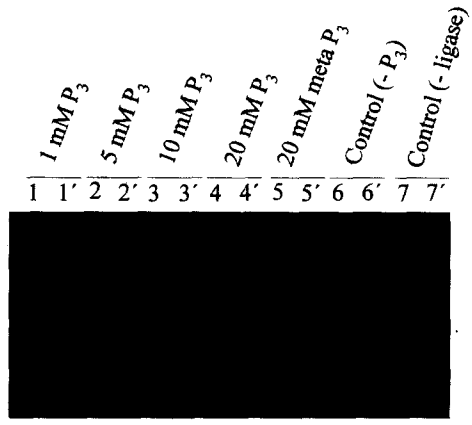

Fig. 5. Ligation of λ DNA/*Hin*dIII fragments catalyzed by T4 DNA ligase. Inhibition by P_3. Reaction mixtures containing λ DNA/*Hin*dIII fragments were incubated in the presence (or absence) of T4 DNA ligase and the compounds indicated. After incubation for 5 min (lanes 1–7) or 15 min (lanes 1′–7′) at 30°C, aliquots of the reaction mixtures were subjected to electrophoresis in a 0.8% agarose gel as indicated in Section 2.

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