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ORIGINAL PAPER

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Thermostable *Pyrococcus furiosus* DNA ligase catalyzes the synthesis of (di)nucleoside polyphosphates

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Abstract DNA ligase from the hyperthermophilic marine archaeon *Pyrococcus furiosus* (*Pfu* DNA ligase) synthesizes adenosine 5′-tetraphosphate (p_4A) and dinucleoside polyphosphates by displacement of the adenosine 5′-monophosphate (AMP) from the *Pfu* DNA ligase-AMP (E-AMP) complex with tripolyphosphate (P_3), nucleoside triphosphates (NTP), or nucleoside diphosphates (NDP). The experiments were performed in the presence of 1–2 μM [α-^{32}P]ATP and millimolar concentrations of NTP or NDP. Relative rates of synthesis (%) of the following adenosine(5′)tetraphospho(5′)nucleosides (Ap_4N) were observed: Ap_4guanosine (Ap_4G) (from GTP, 100); Ap_4deoxythymidine (Ap_4dT) (from dTTP, 95); Ap_4xanthosine (Ap_4X) (from XTP, 94); Ap_4deoxycytidine (Ap_4dC) (from dCTP, 64); Ap_4cytidine (Ap_4C) (from CTP, 60); Ap_4deoxyguanosine (Ap_4dG) (from dGTP, 58); Ap_4uridine (Ap_4U) (from UTP, <3). The relative rate of synthesis (%) of adenosine(5′)triphospho(5′)nucleosides (Ap_3N) were: Ap_3guanosine (Ap_3G) (from GDP, 100); Ap_3xanthosine (Ap_3X) (from XDP, 110); Ap_3cytidine (Ap_3C) (from CDP, 42); Ap_3adenosine (Ap_3A) (from ADP, <1). In general, the rate of synthesis of Ap_4N was double that of the corresponding Ap_3N. The enzyme presented optimum activity at a pH value of 7.2–7.5, in the presence of 4 mM Mg^{2+}, and at 70°C. The apparent K_m values for ATP and GTP in the synthesis of Ap_4G were about 0.001 and 0.4 mM, respectively, lower values than those described for other DNA or RNA ligases. *Pfu* DNA ligase is used in the ligase chain reaction (LCR) and some of the reactions here reported [in particular the synthesis of Ap_4adenosine (Ap_4A)] could take place during the course of that reaction.

Key words Adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) · Dinucleoside polyphosphates · Ligase chain reaction · Ligases · *Pyrococcus furiosus*

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Introduction

We have recently shown that T4 DNA ligase catalyzes the synthesis of dinucleoside polyphosphates (Madrid et al. 1998), a family of compounds acting in a variety of processes at the intra and extracellular levels (Baxi and Vishwanatha 1995; Kisselev et al. 1998; McLennan 1992, 2000). The problem of the synthesis of dinucleoside polyphosphates has been studied in this laboratory since 1990 (Guranowski et al. 1990). It seems that all the ligases tested, including the previously described aminoacyl-tRNA synthetases (Plateau and Blanquet 1992), able to transfer a nucleotidyl moiety via nucleotidyl-containing intermediates and releasing pyrophosphate (PP_i), catalyze the synthesis of dinucleoside polyphosphates if the proper experimental conditions are set up. Examples of these types of enzymes are: firefly luciferase (EC 1.13.12.7) (Ortiz et al. 1993); acetyl-CoA synthetase (EC 6.2.1.1) (Guranowski et al. 1994); acyl-CoA synthetase (EC 6.2.1.3) (Fontes et al. 1998); T4 DNA ligase [adenosine 5′-monophosphate (AMP)-forming] (EC 6.5.1.1) (Madrid et al. 1998) and T4 RNA ligase (EC 6.5.1.3) (Atencia et al. 1999). See also Sillero and Günther Sillero (2000) for a review.

T4 DNA ligase catalyzes the formation of phosphodiester bonds between neighboring 3′-hydroxyl and 5′-phosphate ends in double-stranded nicked DNA (Lindahl and Barnes 1992) (equations 1, 2, 3). Similar reactions and mechanisms have been reported for DNA ligases from thermophilic microorganisms (Kletzin 1992; Luo and Barany 1996; Takahashi et al. 1984).

$$E + ATP \leftrightarrow E - AMP + PP_i \quad (1)$$

$$E - AMP + 5'P - DNA \rightarrow E - AppDNA \quad (2)$$

$$E - AppDNA + 3' - OH - DNA \rightarrow E + DNA - p - DNA + AMP \quad (3)$$

In the presence of pyrophosphatase, and in the absence of DNA, T4 DNA ligase catalyzes the synthesis of adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) [from adenosine 5′-triphosphate (ATP)] or adenosine(5′)tetraphospho(5′)nucleosides (Ap_4N) [from ATP and nucleoside triphosphates (NTP)] (equation 4) or adenosine(5′)triphospho(5′)adenosine (Ap_3A) [from ATP and adenosine 5′-diphosphate (ADP)] (Madrid et al. 1998).

$$E - AMP + NTP \rightarrow Ap_4N + E \quad (4)$$

In this report, we describe the synthesis of (di)nucleoside polyphosphates catalyzed by DNA ligase from the hyperthermophilic marine archaeon *Pyrococcus furiosus* (*Pfu*).

The interest of this work stems from the following considerations: (1) *Pfu* DNA ligase is widely used in the so-called ligase chain reaction (LCR) (Barany 1991; Landegren et al. 1988) where DNA amplification is obtained by a cyclic two-step reaction in which double-stranded target DNA unwinds to become single-stranded, through the effect of high temperature, followed by a cooling step in which two sets of adjacent complementary oligonucleotides anneal to the single-stranded DNA target and are joined by the ligase; this method results in an exponential amplification of the ligation products; (2) to our knowledge, this is the first example of a thermophilic enzyme catalyzing the synthesis of dinucleoside polyphosphates.

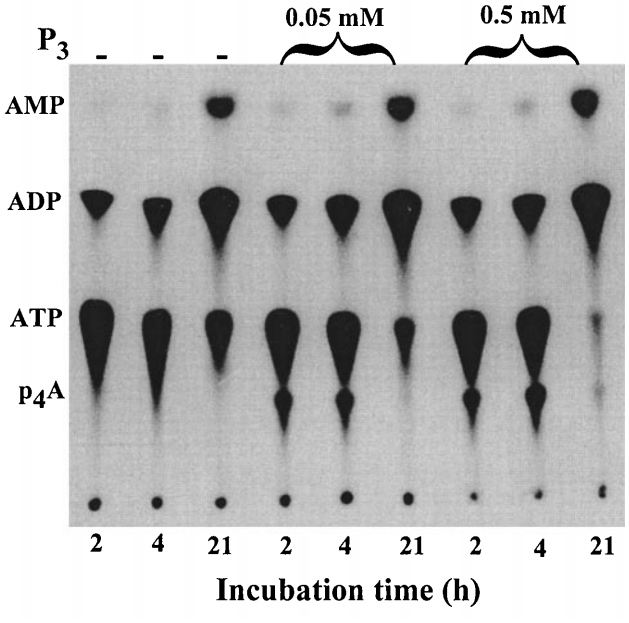

Fig. 1. Synthesis of adenosine 5′-tetraphosphate (p_4A) catalyzed by *Pfu* DNA ligase. The reaction mixtures (0.02 ml) contained 0.002 mM (0.2 µCi) [α-^{32}P]ATP, 4 U enzyme, in the absence or presence of tripolyphosphate (P_3), at the indicated concentrations, and other components as described in the text. Samples were taken after 2-, 4-, and 21-h incubation; subjected to thin-layer chromatography (*TLC*); and the radioactivity measured with an InstantImager (Konica)

Materials and methods

Materials

Pfu DNA ligase was purchased from Stratagene (Cat no 600191; lot numbers, 0680707; 0190708; 0790709). The specific activity of the samples was 11,667 U/mg). One unit (U) will ligate to completion 0.5 µg of nicked Bluescript DNA in 15 min at 55°C. Thermostable inorganic pyrophosphatase (EC 3.6.1.1) was from BioLabs (Cat no 296S, lot number 6). One unit is the amount of enzyme that will generate 40 nmol/min of inorganic phosphate (P_i) from PP_i at 75°C. Alkaline phosphatase (grade I) (EC 3.1.3.1) and phosphodiesterase from *Crotalus durissus* (EC 3.1.4.1) were obtained from Boehringer Mannheim. [α-^{32}P]ATP, 3,000 Ci/mmol, was from Dupont NEN. Thin-layer chromatography (TLC) silica gel fluorescent plates were from Merck. X-ray films were from Konica Corporation (Japan). Radioactively labeled nucleotides were quantified, in conditions of linearity, with the help of an InstantImager (Packard Instrument Company). Asymmetrical dinucleoside tetraphosphatase (EC 3.6.1.17) and dinucleoside triphosphatase (EC 3.6.1.29) were purified from rat liver as previously described (Sillero et al. 1977, 1997).

Pfu DNA ligase-AMP complex formation

The reaction mixture (0.02 ml) contained 25 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 5 µM (0.1 µCi) [α-^{32}P]ATP, 0.2 U thermostable pyrophosphatase and, where indicated, tripolyphosphate (P_3). The mixture was incubated for 15 min at 70°C to remove the potential PP_i contaminating the commercial preparation of P_3. The formation of the *Pfu* DNA ligase-AMP complex (or E-AMP complex) was initiated by the addition of 2 U (0.17 µg) of *Pfu* DNA ligase. After 15 min incubation at 70°C, the reactions were stopped with 6.5 µl of concentrated sodium dodecyl sulfate (SDS) sample buffer [0.25 M Tris/HCl (pH 6.8), 8% SDS, 40% glycerol, 240 mM dithiothreitol (DTT), 0.005% bromophenol blue]. The mixtures were heated at 90°C for 3 min and 10 µl aliquots were loaded onto a 12% denaturing polyacrylamide gel. The gel was stained with Coomassie Blue (a unique band with an apparent molecular mass of 64 kDa was observed) dried down and the labeled enzyme-adenylate complex detected by autoradiography.

Synthesis of (di)nucleoside polyphosphates by *Pfu* DNA ligase

Unless otherwise indicated, the reaction mixtures (0.02 ml) contained 25 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 25 mM KCl; 5 mM $MgCl_2$; 10 U/ml of thermostable pyrophosphatase; 0.002 mM [α-^{32}P]ATP (0.2 µCi); P_3, guanosine 5′-triphosphate (GTP) or other nucleotides as indicated and 4 U of *Pfu* ligase (Fig. 1). The reaction mixtures were overlaid with 0.025 ml of mineral oil and incubated at 70°C. Aliquots (2 µl) of the reactions were taken, at different times of

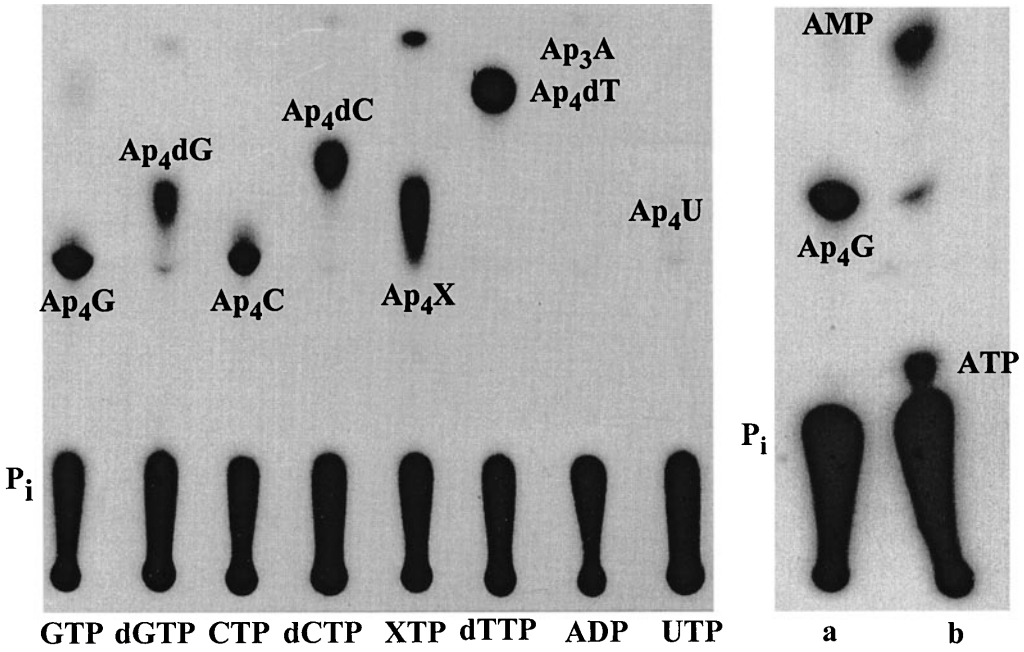

Fig. 2. Synthesis of adenosine(5′)tetraphospho(5′)nucleosides (Ap_4N) by *Pfu* DNA ligase. *Left panel*: the reaction mixture (0.02 ml) contained 0.001 mM (0.2 μCi) [α-^{32}P]ATP, 4 U enzyme, and 1 mM each of guanosine 5′-triphosphate (*GTP*), deoxyguanosine 5′-triphosphate (*dGTP*), cytidine 5′-triphosphate (*CTP*), deoxycytidine 5′-triphosphate (*dCTP*), xanthosine 5′-triphosphate (*XTP*), deoxythymidine 5′-triphosphate (*dTTP*), uridine 5′-triphosphate (*UTP*), or adenosine 5′-diphosphate (*ADP*). After 6-h incubation, 1 μg of alkaline phosphatase was added to each sample and incubated further for 1 h. Samples were taken, subjected to TLC, and the radioactivity measured with an InstantImager (Konica). *Right panel*: the remainder of the reaction mixture (0.01 ml) that contained synthesized adenosine(5′)tetraphospho(5′)guanosine (Ap_4G) was heated at 100°C for 6 min to inactivate alkaline phosphatase (*a*), and further incubated in the presence of 1 mU of purified dinucleoside tetraphosphatase for 15 min at 37°C (*b*)

incubation, spotted on silica gel plates, along with standards, and developed in dioxane : ammonium hydroxide : water 6 : 1 : 5 (Figs. 2a, 3a) or 6 : 1 : 6 by volume (Figs. 1, 2b, 3b). Nucleotide spots were localized with a 253-nm wavelength light and the corresponding radioactivity measured by autoradiography and/or with an InstantImager, in linearity conditions. In some experiments, before TLC analysis, the reaction mixtures were treated with alkaline phosphatase (1 μg for 1 h at 37°C) to allow a better quantification of the dinucleotide polyphosphates synthesized.

Results and discussion

Formation of the E-AMP complex and its reaction with P_3

When *Pfu* ligase was incubated in the absence of DNA and in the presence of [α-^{32}P]ATP, as described earlier, a main E-AMP complex was formed that migrated on SDS-PAGE (SDS-polyacrylamide gel electrophoresis) into a position corresponding to a molecular mass of about 64 kDa. The presence of P_3 in the reaction mixtures inhibited the formation of the E-AMP complex in a concentration-dependent manner, in a similar way to that previously described for T4 DNA and T4 RNA ligases (Madrid et al. 1998; Atencia et al. 1999). In this case, around 33%, 57%, 87%, and 100% inhibition was obtained in the presence of 0.05, 0.1, 0.5, and 1 mM P_3, respectively (results not shown).

In a different experiment, the enzyme was incubated in the presence of 2 μM [α-^{32}P]ATP and two different concentrations of P_3 (0.05 and 0.5 mM). After incubation of the reaction mixtures for 2, 4, and 21 h at 70°C, radioactive spots corresponding to adenosine 5′-tetraphosphate (p_4A) were detected (Fig. 1). These spots were characterized as p_4A due to their sensitivity to alkaline phosphatase and to co-migration with a standard of unlabeled p_4A. After 21 h of incubation, the spots corresponding to ATP and p_4A diminished considerably, whereas those corresponding to ADP and AMP increased greatly (Fig. 1) due to the instability of ATP and p_4A at the temperature of the reaction (70°C). Because of this, in the experiments presented here the incubation time was usually less than 6 h.

Synthesis and characterization of Ap_4N

The synthesis of Ap_4N was approached in the presence of 0.001 mM [α-^{32}P]ATP, enzyme, and 1 mM of the indicated nucleoside 5′-phosphates [GTP, deoxyguanosine 5′-triphosphate (dGTP), cytidine 5′-triphosphate (CTP), deoxycytidine 5′-triphosphate (dCTP), xanthosine 5′-triphosphate (XTP), deoxythymidine 5′-triphosphate (dTTP), uridine 5′-triphosphate (UTP), and ADP]. The reaction mixtures were incubated for 6 h, then treated with alkaline phosphatase in order to remove any residual [^{32}P]-labeled nucleoside 5′-phosphates, and analyzed by TLC as indicated in Fig. 2 (left panel). The radioactive spots

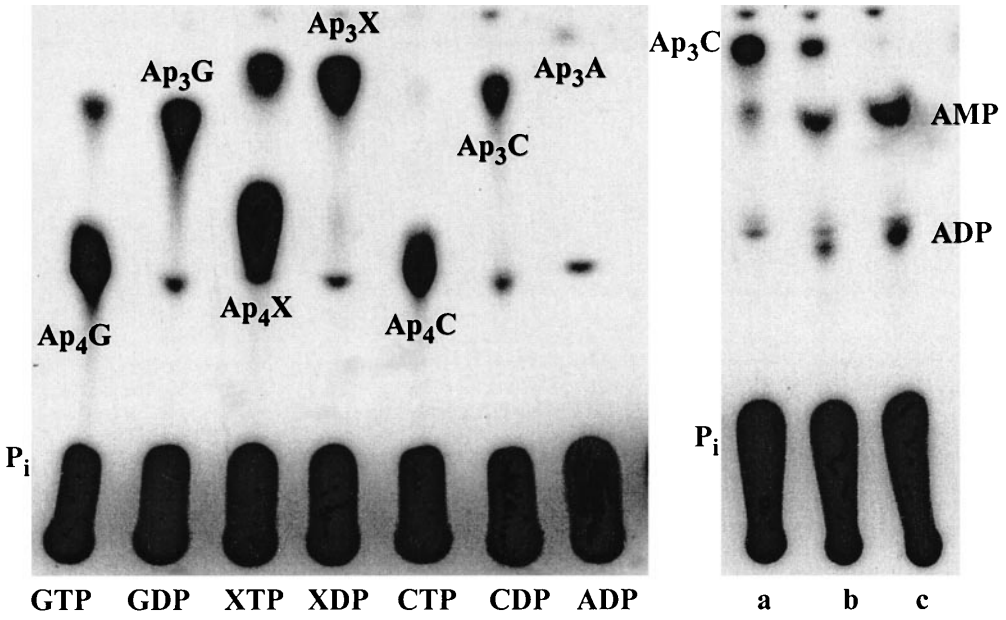

Fig. 3. Comparative synthesis of adenosine(5′)triphospho(5′)nucleosides (*Ap3N*) and Ap4N by *Pfu* DNA ligase. *Left panel*: the reaction mixtures (0.02 ml) contained 0.001 mM (0.2 μCi) [α-^{32}P]ATP, 1 mM nucleoside diphosphates (*NDP*), or nucleoside triphosphates (*NTP*) (as indicated) and 4 U of enzyme (other components as described in the text). After 6-h incubation, 1 μg of alkaline phosphatase was added to each sample and incubated further for 1 h at 37°C. Aliquots were taken and subjected to TLC. *Right panel*: the remainder of the reaction mixture containing synthesized adenosine(5′)triphospho(5′)cytidine (*Ap3C*) was heated at 100°C for 6 min to inactivate alkaline phosphatase (*a*) and further incubated in the presence of 0.1 mU of purified dinucleoside triphosphatase for 5 min (*b*) or 20 min (*c*) at 37°C

at the bottom of the plate correspond to inorganic [^{32}P]. From the results obtained (Fig. 2) the following relative rates of synthesis (%) for the corresponding dinucleotides were calculated: adenosine(5′)tetraphospho(5′)guanosine (Ap_4G) (100); adenosine(5′)tetraphospho(5′)deoxythymidine (Ap_4dT) (95); adenosine(5′)tetraphospho(5′)xanthosine (Ap_4X) (94); adenosine(5′)tetraphospho(5′)deoxycytidine (Ap_4dC) (64); adenosine(5′)tetraphospho(5′)cytidine (Ap_4C) (60); adenosine(5′)tetraphospho(5′)deoxyguanosine (Ap_4dG) (58); adenosine(5′)tetraphospho(5′)uridine (Ap_4U) (<3), and Ap_3A (<1), where 100% represents the synthesis of 2.0 nmol of Ap_4G/h per milligram of protein.

The identity of these dinucleotides was assessed by their insensitivity to alkaline phosphatase, by their coelution with standards (when available) and by treatment with dinucleoside tetraphosphatase. As an example, the results obtained after treatment of labeled synthesized Ap_4G (Ap*pppG) with purified asymmetrical dinucleoside tetraphosphatase are shown in Fig. 2 (right panel). As expected, labeled AMP and ATP were obtained. The other products of the reaction, unlabeled GTP and guanosine 5′-monophosphate (GMP), were not observed.

Synthesis and characterization of adenosine(5′)triphospho(5′)nucleosides

In spite of the results presented in Fig. 2, in which very low (if any) synthesis of labeled Ap_3A was obtained from ATP and ADP, the synthesis of adenosine(5′)triphospho(5′)nucleosides (Ap_3N) was done similarly to that of Ap_4N by incubating the enzyme in the presence of 0.001 mM [α-^{32}P]ATP and 1 mM of each of the following pairs of NTP and nucleoside diphosphate (NDP) nucleotides: GTP, guanosine 5′-diphosphate (GDP); XTP, xanthosine 5′-diphosphate (XDP); CTP, cytidine 5′-diphosphate (CDP); and ADP. The reaction mixtures were incubated for 6 h at 70°C followed by treatment with alkaline phosphatase (Fig. 3). Confirming the results presented in Fig. 2, GTP and XTP were better substrates than CTP for the synthesis of the corresponding Ap_4N; a synthesis of adenosine(5′)triphospho(5′)guanosine (Ap_3G) and adenosine(5′)triphospho(5′)xanthosine (Ap_3X) could also be observed, but was very slow; this was due to the NDPs contaminating the commercial preparations of GTP and XTP. The relative rates of synthesis (%) of Ap_3N in the presence of GDP, XDP, CDP, or ADP were: Ap_3X (110); Ap_3G (100); adenosine(5′)triphospho(5′)cytidine (Ap_3C) (42); Ap_3A (<1), where 100% represents the synthesis of 1 nmol of Ap_3G/h per milligram of protein. In the same experimental conditions, the amount of Ap_3N synthesized is around 50% of the corresponding Ap_4N.

The identity of Ap_3N was assessed by its insensitivity to alkaline phosphatase and by treatment with dinucleoside triphosphatase. As an example, Fig. 3 (right panel) shows the treatment of labeled synthesized Ap_3C (Ap*ppC) with purified dinucleoside triphosphatase; as expected, labeled AMP and ADP were obtained; the other two products of the reaction were unlabeled CDP and cytidine 5′-monophosphate (CMP).

Synthesis of Ap_4A was observed in experiments using a higher concentration of [α-^{32}P]ATP (0.02 mM). In the pres-

ence of both 0.02 mM GTP and 0.02 mM [α-^{32}P]ATP, synthesis of labeled Ap_4G and Ap_4A took place at a similar rate.

Activation by pyrophosphatase

The rate of synthesis of Ap_4G was stimulated by the presence of pyrophosphatase (PPase) in the reaction mixture. The initial rates of synthesis of Ap_4G, in the presence and absence of PPase, were 0.5 and 1.6 nmol/h per milligram of protein, respectively. Similar results were obtained when the synthesis of p4A (from ATP and P_3) was carried out in the presence or absence of pyrophosphatase (results not shown).

Effects of pH, metals, and temperature on the reaction rate

The effect of pH on the rate of synthesis of a dinucleoside tetraphosphate (Ap_4G) was determined in reaction mixtures containing 0.001 mM [α-^{32}P]ATP, 1 mM GTP, enzyme, and 25 mM Tris/HCl buffer at various pH values (6.5–7.9). Maximal reaction rates (2.2 nmol/h per milligram) were measured at pH values of 7.2–7.5. At pH 6.5 and 7.9, the enzyme activity was 25% and 75% of the maximum, respectively. Similar values were found for T4 DNA ligase (unpublished results) and T4 RNA ligase (Atencia et al. 1999).

The rate of synthesis of Ap_4G was determined, in standard reaction mixtures, incubating at 70°, 50° and 37°C for 4 h. The synthesis was highest at 70°C (100%, or 2.1 nmol/h per milligram), and decreased to 26% at 50°C and to 11% at 37°C.

Pfu ligase is a Mg-dependent enzyme. The effect of Mg^{2+} concentration on the synthesis of Ap_4G was assayed in standard reaction mixtures, containing 1 mM GTP and in the presence or absence of 1, 2, or 4 mM $MgCl_2$, respectively. No synthesis was observed in the absence of Mg^{2+} and relative rates of 47%, 79%, and 100% were obtained at 1, 2, and 4 mM Mg concentration, respectively. Similar requirement for Mg^{2+} have been found for RNA and DNA ligases from T4 (unpublished results).

K_m values for ATP and GTP in the synthesis of Ap_4G

The synthesis of dinucleoside polyphosphates catalyzed by ligases (Plateau and Blanquet 1992) follows two steps: (1) formation of the E-acyl-AMP (or E-AMP) complex; (2) transfer of the AMP moiety of that complex to a nucleoside triphosphate (NTP) with formation of Ap_4N. In general, the K_m values for ATP in the first step, and for NTP in the second step are in the order of micromolar and millimolar, respectively. Using *Pfu* DNA ligase, the K_m values determined for ATP and GTP in the synthesis of Ap_4G were 1 μM and 0.4 mM, respectively. This last value is around three times lower than the corresponding values determined for T4 DNA ligase (Madrid et al. 1998) and T4 RNA ligase (Atencia et al. 1999). In those experiments a rate of synthesis (k_{cat}) of around 1.2×10^{-4} s^{-1} was calculated for Ap_4G. This value is around 70 and 40 times lower than those determined for T4 RNA ligase (Atencia et al. 1999) and T4 DNA ligase (unpublished results), respectively.

Conclusions

As stated in the Introduction, it is now well established that ligases catalyze the synthesis of dinucleoside polyphosphates by different mechanisms involving formation of either an E-acyl-AMP complex (firefly luciferase) or an E-AMP complex without need of an acid residue (T4 DNA ligase and T4 RNA ligase) (Sillero and Günther Sillero 2000). What is common to all these mechanisms is the liberation of PP_i in the first step of the reaction (reaction 1). From a thermodynamic point of view, reactions of this type are reversible because the energy contents of substrates and products are similar. The capacity of an enzyme with these characteristics to catalyze the synthesis of dinucleoside polyphosphates would depend on the specificity of the PP_i site of the enzyme toward the alternative substrate (NTP or NDP); hence, the synthesis of dinucleoside polyphosphates could be envisaged as the reversal of the reaction of formation of PP_i (compare reactions 1 and 4). From the above, it is clear that the elimination of PPi, by the addition of extra inorganic pyrophosphatase, would favor both the accumulation of E-AMP and its reaction with NTP or NDP to form Ap_4N or Ap_3N. In the case of the *Pyrococcus* DNA ligase, NTP seems to react with the E-AMP complex more efficiently than NDP for the formation of the corresponding dinucleotide, as the rate of synthesis of Ap_4N is approximately double that of Ap_3N.

Although it is unusual, and probably also difficult, to quantify some of the enzymes used in genetic engineering in terms of classical enzyme kinetic units, we thought it would be interesting to try to correlate the *Pfu* DNA ligase activity described here with its DNA ligation activity. According to the data provided by Stratagene, one unit of ligase (containing around 0.857×10^{-7} g or 1.34×10^{-12} mol of enzyme) will ligate 0.5 μg of Bluescript DNA (2,961 bp), or 2.8×10^{-13} mol (assuming a molecular weight for the phagemid of $2{,}961 \times 2 \times 300 = 1.77 \times 10^6$ g) to completion in 15 min. Assuming that the Bluescript DNA to be linked contains one nick per molecule, an approximate k_{cat} value of 2.2×10^{-4} s^{-1} was calculated; a figure similar to that determined for the synthesis of Ap_4G ($k_{cat} = 1.2 \times 10^{-4}$ s^{-1}). Considering the intracellular concentrations of ATP, NTP, and of the potentially nicked DNA, it seems that the synthesis of dinucleoside polyphosphates is not a marginal activity in relation to the physiological role assigned to this enzyme as a DNA ligase.

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