Firefly luciferase synthesizes P^1,P^4-bis(5′-adenosyl)tetraphosphate (Ap_4A) and other dinucleoside polyphosphates+

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The synthesis of P^1,P^4-bis(5′-adenosyl)tetraphosphate (Ap_4A) has been considered, for a long time, to be catalyzed mainly by some aminoacyl-tRNA synthetases [Brevet et al. (1989) Proc. Natl. Acad. Sci. USA 86, 8275–8279]. Recently, yeast Ap_4A phosphorylase, acting in reverse (Guranowski et al. (1988) Biochemistry 27, 2959–2964), was shown to synthesize Ap_4A, too. In the case of the synthetases, the intermediate complex E-aminoacyl-AMP may serve as donor of AMP to ATP, yielding Ap_4A. Here we demonstrate that firefly luciferase (EC 1.13.12.7) which forms the E-luciferin-AMP intermediate also synthesizes Ap_4A as well as other dinucleoside polyphosphates. We suggest moreover that: other enzymes (mainly synthetases and some transferases), which catalyze the transfer of a nucleotidyl moiety, via nucleotidyl-containing intermediates and releasing PP_i may produce dinucleoside polyphosphates.

Diadenosine tetraphosphate; Dinucleoside polyphosphate; Firefly luciferase; Pyrophosphate; Synthetase

1. INTRODUCTION

Diadenosine 5′,5′′′-P^1,P^4-tetraphosphate (Ap_4A) occurs in procaryotes [1–3] and eucaryotes [3–7] at the picomolar–micromolar range. This nucleotide has been implicated in the control of cell division [7,8], and found to be a ligand of eucaryotic DNA polymerase α [9], an inhibitor of some reactions [10,11], and an activator of poly(ADP-ribose) polymerase [12], AMP deaminase [13] and cytosolic 5′-nucleotidase [14]. Ap_4A has also been considered as an alarmone as its concentration, and that of other dinucleoside polyphosphates (DNPPs), increases at stress situations [2,3,15]. The level of Ap_4A results from the rates of the nucleotide synthesis and degradation. Several specific [16–21] and unspecific [20–23] enzymes cleave Ap_4A. So far, the bulk of the synthesis of Ap_4A was believed to depend on some aminoacyl-tRNA synthetases, catalyzing reactions (A1) and (A2).

$$\text{aminoacid} + \text{ATP} + \text{E} \longrightarrow \text{E-aminoacyl-AMP} + PP_i \quad \text{(A1)}$$

$$\text{E-aminoacyl-AMP} + \text{ATP} \longrightarrow Ap_4A + \text{aminoacid} + \text{E} \quad \text{(A2)}$$

Since firefly luciferase (EC 1.13.12.7) catalyzes reaction (A3), which is similar to reaction (A1), we wondered if the enzyme could perform also reaction (A4).

$$\text{luciferin} + \text{ATP} + \text{E} \longrightarrow \text{E-luciferin-AMP} + PP_i \quad \text{(A3)}$$

$$\text{E-luciferin-AMP} + \text{ATP} \longrightarrow Ap_4A + \text{luciferin} + \text{E} \quad \text{(A4)}$$

The results shown below confirmed our expectations and allow us to present a more general view, so far lacking, on the metabolism and function of dinucleoside polyphosphates.

2. MATERIALS AND METHODS

2.1. *Synthesis of diadenosine tetraphosphate by firefly luciferase*

The stock solution of firefly (*Photinus pyralis*) luciferase (Sigma Cat. No. L5226 Lot 106F-8065) was prepared dissolving 1 mg of the preparation in 0.25 ml of a buffer containing 100 mM Hepes-KOH (pH 7.5), 1 mM dithiothreitol, 5% glycerol and 0.25 mg bovine serum albumin. The reaction mixture for the synthesis of Ap_4A contained the following in a final volume of 0.1 ml: 50 mM Hepes-KOH buffer, pH 7.5, 5 mM $MgCl_2$, 2.5 mM ATP, 0.25 mM D-luciferin (Sigma), 5 U of inorganic pyrophosphatase from yeast (Boehringer) and luciferase (0.01 ml (35 mg of protein) of the stock solution). After incubation at 30°C for different times, aliquots were withdrawn from the reaction mixture and analyzed by either high-pressure liquid chromatography (Figs 1 and 2) or thin-layer chromatography (Fig. 3). In the first case, 0.01 ml aliquots were withdrawn, transferred into 0.14 ml of hot water and kept at 95°C for 2 min. After chilling, the mixture was filtered through a nitrocellulose membrane (Millipore HA 0.45 mm) and a 0.05 ml portion injected into a Hypersil ODS col-

+ To Prof. Alberto Sols, in memoriam

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Abbreviations: Ap_4A, diadenosine 5′,5′′′-P^1,P^4-tetraphosphate or diadenosine tetraphosphate; Ap_4G, adenosine 5′-P^1-tetraphospho-P^4-5′′′-guanosine; Gp_4G, diguanosine 5′,5′′′-P^1,P^4-tetraphosphate or diguanosine tetraphosphate; Np_4N, dinucleoside tetraphosphate; DNPP, dinucleoside polyphosphate; NMP, nucleoside monophosphate; X, organic acid able to form an anhydride bond with NMP

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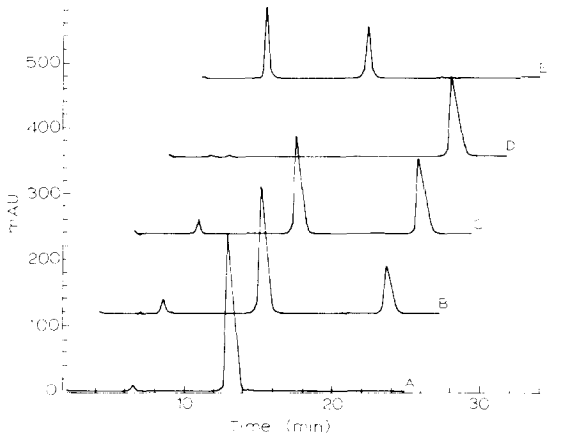

Fig. 1. Synthesis of diadenosine tetraphosphate by firefly luciferase. The reaction mixture was as described in Materials and Methods. Aliquots (0.01 ml) were withdrawn after 0, 60 and 120 min of incubation and subjected to HPLC (chromatogram A, B, and C, respectively). Chromatograms D and E represent the characterization of Ap_4A as one of the products of the reaction and were obtained as follows: after withdrawal of the last sample, the reaction was stopped by heating the remaining mixture at 95°C for 2 min, cooled to 30°C and supplemented with 1 μl of the suspension of calf intestine alkaline phosphatase (about 10 units) (Boehringer, Grade I), in order to remove nucleotides with terminal phosphates. After 30 min of incubation, an 0.01-ml aliquot was withdrawn and subjected to HPLC as described in Materials and Methods (profile D). The remaining mixture was heated again at 95°C for 2 min to inactivate the phosphatase, cooled at 30°C, brought to 5 mM K_2HPO_4 and supplemented with 1 μl (0.5 units) of the yeast diadenosine tetraphosphate phosphorylase (purified as described in [19]). The reaction was allowed to proceed for 30 min and an 0.01-ml aliquot was taken for chromatography (profile E). For a better comparison of the reaction products, the chromatograms shown cover only the elution profiles between 2 and 25 min. Because of that, the peaks corresponding to nucleosides which eluted around 1 min in chromatograms D and E are not seen. Standards of ADP, ATP and Ap_4A, run in the same conditions eluted at 6.4, 12.9 and 21.4 min, respectively.

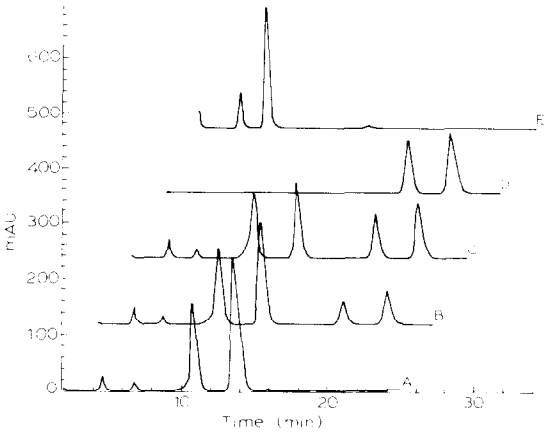

Fig. 2. Synthesis of Ap_4A and Ap_4G by firefly luciferase. Experimental procedures were as described in Material and Methods except that the reaction mixture was supplemented with 2.5 mM GTP, and the reaction products (Ap_4A and Ap_4G) were subjected to the action of the (symmetrical) dinucleoside tetraphosphatase from *E. coli* [18]. After inactivating the alkaline phosphatase (see Fig. 1), the mixture was brought to 0.2 mM $CoCl_2$ and supplemented with 5 μl (0.1 units) of the tetraphosphatase. Profiles A, B and C represent chromatograms of the reaction mixtures after 0, 60 and 120 min of incubation; chromatogram D was obtained after treatment of the luciferase reaction mixture with alkaline phosphatase, and chromatogram E was obtained after hydrolysis of the remaining dinucleotides with the specific tetraphosphatase. Peaks at 4.5, 6.4, 10.3, 13.1, 18.6 and 21.6 min correspond to GDP, ADP, GTP, ATP, Ap_4G and Ap_4A, respectively.

umn (100 × 2.1 mm). Elution was performed at a constant 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, 20% methanol, followed by a 10 min linear gradient (30–100 mM) of sodium phosphate (pH 7.5) in 20 mM tetrabutylammonium, 20% methanol. The HPLC equipment was composed of an HP Liquid Chromatograph (model 1090), with a diode array detector, commanded by an HPLC ChemStation.

3. RESULTS AND DISCUSSION

The synthesis of Ap_4A catalyzed by the firefly luciferase was strictly dependent on luciferin, and one of the following divalent cations: Mg^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+}. The reaction was greatly enhanced in the presence of pyrophosphatase, that precludes reverse of the reaction (A3) (Fig. 3). Under these conditions, the rate of synthesis of Ap_4A by the luciferase was linear (Fig. 1A–C) and similar to that of pyrophosphate liberation when the enzyme activity was measured in the absence of pyrophosphatase (results not shown). If, in addition to ATP, another nucleoside triphosphate (GTP) was added to the reaction mixture, the synthesis of both Ap_4A and adenosine 5′-P^1-tetraphospho-P^4-5′′′-guanosine (Ap_4G) was observed (Fig. 2A–C), thus showing that GTP accepts the adenylyl moiety of the complex E-luciferin-AMP. Identity of Ap_4A (retention time, 21.4 min in Fig. 1) was confirmed by the following criteria: co-chromatography with a standard of commercial Ap_4A in the two chromatographic systems used, spectral characteristics, resistance to alkaline phosphatase (Fig. 1D) and susceptibility to the specific Ap_4A phosphorylase from yeast [19], which produces ADP and ATP (Fig. 1E). Ap_4A and Ap_4G (Fig. 2) were characterized by their spectra, resistance to alkaline phosphatase (Fig. 2D) and by the nature of the products (ADP and GDP) resulting from the treatment of the reaction mixture with the (symmetrical) Ap_4A hydrolase from *Escherichia coli* [18] (Fig. 2E).

Two types of comments can be raised from these results, one of them referring to the luciferase itself and the other one to the metabolism and function of dinucleoside polyphosphates. The synthesis of DNPPs catalyzed by the firefly luciferase, described here for the first time, allows to have a new vision both on the mechanism of reaction and on the physiological function of this enzyme [26]. Interestingly, inorganic

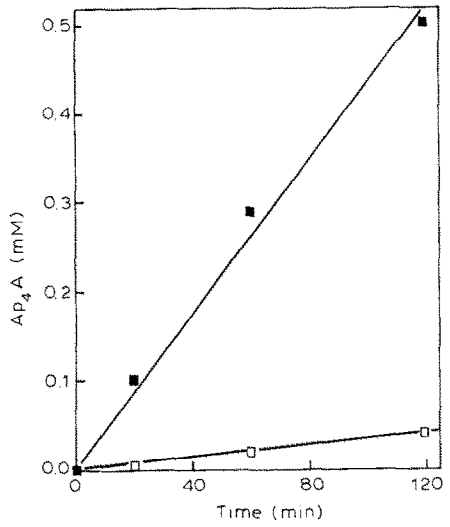

Fig. 3. Effect of pyrophosphatase on the synthesis of diadenosine tetraphosphate catalyzed by firefly luciferase. The reaction mixture contained in a final volume of 0.05 ml the same components as in Material and Methods, plus 1 μCi of [2-8^{3}H]ATP (from Amersham, specific activity 44 Ci/mmol) (■). The same reaction was followed in parallel in the absence of pyrophosphatase (□). At the indicated times, 3 μl aliquots were spotted onto thin layer chromatography plates (from Merck) precoated with silica gel containing a fluorescent indicator. Standard of Ap_4A was applied at the origins and the plates were developed for 90 min in dioxane/ammonia/water (6/1/4, v/v). In this system, the R_f values for ATP, ADP, Ap_4A, and AMP are 0.14, 0.24, 0.60, and 0.65, respectively. Spots corresponding to Ap_4A were cut out, and the radioactivity counted.

pyrophosphatase, which promotes in vitro the synthesis of Ap_4A, is present in very high concentrations in crude extracts of firefly lanterns [26]. If both enzymes are present in the same cellular compartment, it would be worthy to explore the relationships between light emission and the synthesis of Ap_4A in this biological system. Paradoxically, luciferase is widely used for the evaluation of dinucleoside polyphosphates [3,4,25]. In these assays, the hydrolysis of DNPPs by snake venom phosphodiesterase is coupled to the luciferase, either directly [3,4,25] (in the case of Ap_4N), or indirectly [3,4] (in the case of Np_4Ns) through other coupled enzymatic reactions that produce ATP. The effect described here must be taken into account if one applies the bioluminescence assay to the evaluation of DNPPs.

Based on the similarity observed between the reactions catalyzed by the aminoacyl-tRNA synthetases and the luciferase, we suggest that the synthesis of DNPPs could be a quite common phenomenon. Other enzymes which catalyze the transfer of nucleoside monophosphates (AMP or other NMPs), with concomitant formation of PP_i and the intermediate complex E-X-NMP (where X stands for an organic acid able to form an anhydride bond with a NMP) could also be potentially able to synthesize either hetero- or homodinucleoside polyphosphates (with different or the same nucleosides, respectively). Examples of such enzymes are many of the ligases belonging to the subclasses EC 6.1, EC 6.2 or EC 6.3, and some transferases, such as those which transfer CMP (EC 2.7.7.14, EC 2.7.7.15, etc.), UMP (EC 2.7.7.9, EC 2.7.7.10, etc.), GMP (EC 2.7.7.45) [27] or AMP (EC 2.7.7.53) [28]. The synthesis of various DNPPs have been demonstrated with the two latter enzymes [27,28]. In both cases, plausible intermediates of the type E-NMP may participate in the synthesis of DNPPs. In this regard two considerations can be forwarded: (a) the nucleotidyl derivative of the enzyme may act directly as a donor of the nucleotidyl moiety of DNPPs; and (b) some of the transferases may participate in the synthesis of nonadenylylated DNPPs, shown to occur in yeast and *E. coli* [3]. Although the luciferase is classified as an oxidoreductase (EC 1.13.12.7), it behaves also as a ligase, as has been shown here. The molecular uniformity among the reactions catalyzed by aminoacyl-tRNA synthetases, acyl-CoA synthetases and firefly luciferase was already observed by McElroy et al. [29]. However, they did not consider at that time the possibility that luciferase could synthesize Ap_4A. Some of the enzymes mentioned above may contribute to the increased levels of DNPPs observed in cells subjected to stress due to the following factors which, besides the availability of the nucleotides, affect the synthesis of DNPPs. When the normal metabolic equilibria are imbalanced, the level of such cosubstrates as specific tRNAs or CoA, for aminoacyl-tRNA synthetases and acetyl-CoA synthetase, respectively, may decrease favouring the synthesis of DNPPs through reactions between the complex E-X-NMP and different nucleotides. Moreover, these reactions could be promoted if the level of pyrophosphate is still lower in stress than in normal situations. Thus, it is possible that the rate of synthesis of DNPPs depends also on the concentration of compounds not directly involved in the metabolism of the nucleotides. At any rate, the dinucleoside polyphosphates could play both the role of signals indicating a metabolic imbalance and of regulators correcting such situations. Related to that, both Ap_4A and Gp_4G have been suggested to modulate the cellular pools of the adenine and guanine nucleotides, respectively [30].

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