

## Synthesis of dinucleoside polyphosphates catalyzed by firefly luciferase

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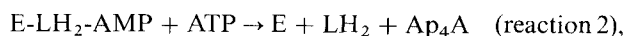
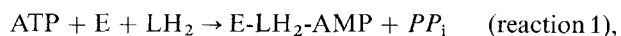
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In the presence of ATP, luciferin (LH<sub>2</sub>), Mg<sup>2+</sup> and pyrophosphatase, the firefly (*Photinus pyralis*) luciferase synthesizes diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate (Ap<sub>4</sub>A) through formation of the E-LH<sub>2</sub>-AMP complex and transfer of AMP to ATP. The maximum rate of the synthesis is observed at pH 5.7. The K<sub>m</sub> values for luciferin and ATP are 2–3 μM and 4 mM, respectively. The synthesis is strictly dependent upon luciferin and a divalent metal cation. Mg<sup>2+</sup> can be substituted with Zn<sup>2+</sup>, Co<sup>2+</sup> or Mn<sup>2+</sup>, which are about half as active as Mg<sup>2+</sup>, as well as with Ni<sup>2+</sup>, Cd<sup>2+</sup> or Ca<sup>2+</sup>, which, at 5 mM concentration, are 12–20-fold less effective than Mg<sup>2+</sup>. ATP is the best substrate of the above reaction, but it can be substituted with adenosine 5'-tetrphosphate (p<sub>4</sub>A), dATP, and GTP, and thus the luciferase synthesizes the corresponding homo-dinucleoside polyphosphates: diadenosine 5',5'''-P<sup>1</sup>,P<sup>5</sup>-pentaphosphate (Ap<sub>5</sub>A), dideoxyadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate (dAp<sub>4</sub>dA) and diguanosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate (Gp<sub>4</sub>G). In standard reaction mixtures containing ATP and a different nucleotide {p<sub>4</sub>A, dATP, adenosine 5'-[α,β-methylene]-triphosphate, (Ap[CH<sub>2</sub>]pp), (S)-adenosine-5'-[α-thio]triphosphate ((Sp)ATP[αS]) and GTP}, luciferase synthesizes, in addition to Ap<sub>4</sub>A, the corresponding hetero-dinucleoside polyphosphates, Ap<sub>5</sub>A, adenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphodeoxyadenosine (Ap<sub>4</sub>dA), diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[α,β-methylene] tetrphosphate (Ap[CH<sub>2</sub>]pppA), (S<sub>p</sub>-diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[α-thio]tetrphosphate ((Sp)Ap<sub>4</sub>A[αS]) and adenosine-5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphoguanosine (Ap<sub>4</sub>G), respectively. Adenine nucleotides, with at least a 3-phosphate chain and with an intact α-phosphate, are the preferred substrates for the formation of the enzyme-nucleotidyl complex. Nucleotides best accepting AMP from the E-LH<sub>2</sub>-AMP complex are those which contain at least a 3-phosphate chain and an intact terminal pyrophosphate moiety. ADP or other NDP are poor adenylate acceptors as very little diadenosine 5',5'''-P<sup>1</sup>,P<sup>3</sup>-triphosphate (Ap<sub>3</sub>A) or adenosine-5',5'''-P<sup>1</sup>,P<sup>3</sup>-triphosphonucleosides (Ap<sub>3</sub>N) are formed. In the presence of NTP (excepting ATP), luciferase is able to split Ap<sub>4</sub>A, transferring the resulting adenylate to NTP, to form hetero-dinucleoside polyphosphates. In the presence of PP<sub>i</sub>, luciferase is also able to split Ap<sub>4</sub>A, yielding ATP. The cleavage of Ap<sub>4</sub>A in the presence of P<sub>i</sub> or ADP takes place at a very low rate. The synthesis of dinucleoside polyphosphates, catalyzed by firefly luciferase, is compared with that catalyzed by aminoacyl-tRNA synthetases and Ap<sub>4</sub>A phosphorylase.

We have recently reported [1] that firefly luciferase catalyzes the synthesis of diadenosine tetrphosphate (Ap<sub>4</sub>A) according to the following equations:



Reaction (3) proceeds faster when, in addition to ATP, Mg<sup>2+</sup> and luciferin, the reaction mixture is supplemented with pyrophosphatase.

The synthesis of Ap<sub>4</sub>A and other dinucleoside polyphosphates (DNPP) by luciferase may have a physiological role, since these compounds probably act as signal molecules. There are reports in favor of [2] and against [3] for the role of Ap<sub>4</sub>A in the control of cell proliferation and DNA replication. Ap<sub>4</sub>A and diguanosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate (Gp<sub>4</sub>G)

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Abbreviations. Ap<sub>3</sub>A, diadenosine 5',5'''-P<sup>1</sup>,P<sup>3</sup>-triphosphate; Ap<sub>4</sub>A, diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate; Ap<sub>5</sub>A, diadenosine 5',5'''-P<sup>1</sup>,P<sup>5</sup>-pentaphosphate; Ap<sub>4</sub>dA, adenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphodeoxyadenosine; dAp<sub>4</sub>dA, dideoxyadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate; Ap<sub>4</sub>G, adenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphoguanosine; Ap<sub>5</sub>G, adenosine 5',5'''-P<sup>1</sup>,P<sup>5</sup>-pentaphosphoguanosine; Ap<sub>3</sub>N, adenosine 5',5'''-P<sup>1</sup>,P<sup>3</sup>-triphosphonucleoside; Ap<sub>4</sub>N, adenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphonucleoside; Ap[CH<sub>2</sub>]pppA, diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[α,β-methylene] tetrphosphate; App[CH<sub>2</sub>]ppA, diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[β,γ-methylene] tetrphosphate; (Sp)Ap<sub>4</sub>A- [αS], (Sp)-diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[α-thio]tetrphosphate; Ap<sub>4</sub>A-

[βS], diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[β-thio]tetrphosphate; DNPP, dinucleoside polyphosphate; Gp<sub>4</sub>G, diguanosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate; Np<sub>3</sub>N, dinucleoside 5',5'''-P<sup>1</sup>,P<sup>3</sup>-triphosphate; Np<sub>4</sub>N, dinucleoside 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate; Ap[CH<sub>2</sub>]pp, adenosine 5'-[α,β-methylene]triphosphate; App[CH<sub>2</sub>]p, adenosine 5'-[β,γ-methylene]triphosphate; (Sp)-ATP[αS], (S)-adenosine-5'-[α-thio]triphosphate; ATP[γS], adenosine-5'-[γ-thio]triphosphate; p<sub>4</sub>A, adenosine 5'-tetrphosphate; p<sub>4</sub>G, guanosine 5'-tetrphosphate; LH<sub>2</sub>, luciferin.

Enzymes. Adenylate kinase (EC 2.7.4.3); alanyl-tRNA synthetase (EC 6.1.1.7); Ap<sub>4</sub>A phosphorylase (EC 2.7.7.53); firefly luciferase (EC 1.13.12.7); inorganic pyrophosphatase (EC 3.6.1.1); lysyl-tRNA synthetase (EC 6.1.1.7); phenylalanyl-tRNA synthetase (EC 6.1.1.20).

may participate in the control of the interconversion of purine nucleotides [4]. Ap<sub>4</sub>A also seems to be involved in the onset of heat shock and oxidative stress [5] and in the function of platelets [6]. Moreover, DNPPs are inhibitors (as mono, or bisubstrate analogs) of a number of transfer reactions in which one or more nucleotides participate [7].

The level of DNPPs results from their rates of synthesis and degradation. Several specific and nonspecific enzymes cleave DNPPs [8, 9]. As recently suggested [1], all the enzymes that catalyze the transfer of a nucleotidyl moiety via nucleotidyl-containing intermediates and releasing PP<sub>i</sub>, may be considered as candidates for the synthesis of DNPPs, as exemplified by the aminoacyl-tRNA synthetases [10–17] and more recently by luciferase [1]. Reactions (2) and (3) had not been previously described for luciferase in spite of extensive studies carried out on the interchange between the adenylyl moiety of the E-LH<sub>2</sub>-AMP complex and ATP or PP<sub>i</sub> [18, 19], and the common use of luciferase to measure ATP and Ap<sub>4</sub>A [20, 21]. Here we present the kinetic characteristics of the synthesis of Ap<sub>4</sub>A and other DNPPs by luciferase and compare these reactions with others reported previously for some aminoacyl-tRNA synthetases [10–17] and Ap<sub>4</sub>A phosphorylase [22].

## MATERIALS AND METHODS

### Materials

**Enzymes.** Luciferase from firefly (*Photinus pyralis*) was purchased from Sigma (Cat. No. L-5226; Lot 36F-8035). The chromatographically prepared, crystallized and lyophilized powder contained 74% protein with an activity of 6.3 U/mg protein. One unit of this enzyme (as defined by Sigma) is the amount of enzyme able to produce 1 nmol pyrophosphate/min at 25°C, in the presence of 0.6 mM ATP and 0.1 mM D-luciferin. Inorganic pyrophosphatase from yeast was from Boehringer. 1 U is the amount of enzyme able to hydrolyze 1 μmol PP<sub>i</sub>/min at 25°C.

**Chemicals.** ATP, GTP, dATP, adenosine 5'-[α,β-methylene]triphosphate (Ap[CH<sub>2</sub>]pp), adenosine 5'-[β,γ-methylene]triphosphate (App[CH<sub>2</sub>]p) and D-luciferin were from Sigma; (S)-adenosine-5'-[α-thio]triphosphate ((Sp)ATP[αS]) and adenosine-5'-[γ-thio]triphosphate (ATP[γS]) were from Boehringer. The TLC plates, coated with silica gel and containing a fluorescent indicator, were from Merck (Art. 5554).

### Methods

**Luciferase assay.** The stock solution of firefly (*P. pyralis*) luciferase was prepared by dissolving 2 mg powder in 0.3 ml 25 mM Hepes/KOH, pH 7.1, 1 mM dithiothreitol, 10% glycerol and 1 mg/ml bovine serum albumin. The standard reaction mixture for the synthesis of Ap<sub>4</sub>A contained the following in a final volume of 0.05 ml: 50 mM Hepes/KOH, pH 7.5, 5 mM MgCl<sub>2</sub>, 2.5 mM ATP, 0.1 mM D-luciferin, 0.05 U inorganic pyrophosphatase and luciferase (25 μg protein). After incubation at 30°C for different times, aliquots were withdrawn from the reaction mixture and analyzed by either HPLC or TLC. In the first case, 0.015-ml aliquots were withdrawn, transferred into 0.185 ml hot water and kept at 95°C for 1.5 min. After chilling, the mixture was filtered through a nitrocellulose membrane (Millipore HA, 0.45 μm) and a 0.05-ml portion injected into a Hypersil ODS column (100 mm × 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)

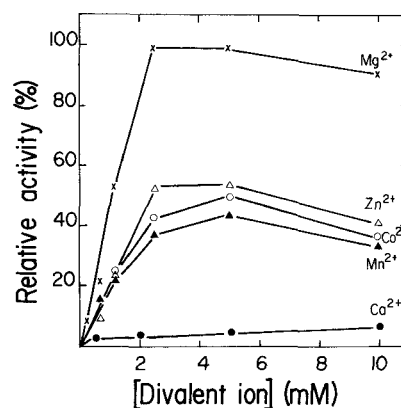


Fig. 1. Dependence of the initial rate of Ap<sub>4</sub>A synthesis, catalyzed by firefly luciferase, on metal-ion concentration. The incubation mixture (50 μl), buffered with 50 mM Mes/KOH, pH 5.7, contained 0.1 mM luciferin, 2.5 mM [8-<sup>14</sup>C] ATP (0.2 μCi), 0.05 U inorganic pyrophosphatase, various concentrations of metals, in the form of chloride and luciferase (28 μg protein). To estimate initial rates, 3 μl aliquots were withdrawn after 6, 12, 18 and 30 min of incubation and analyzed by TLC. Initial velocities are expressed as percentages of that observed at 2.5 mM MgCl<sub>2</sub>.

of sodium phosphate, pH 7.5 in 20 mM tetrabutylammonium bromide/20% methanol, followed by a 10 min linear gradient (30–100 mM) of sodium phosphate, pH 7.5, in 20 mM tetrabutylammonium bromide/20% methanol. The concentration of each nucleotide in the sample was estimated by comparison with the chromatogram of standard solutions of known nucleotide concentration. In the case of diadenosine polyphosphates other than Ap<sub>4</sub>A, the standard calibration curve corresponding to the latter compound was used. The HPLC equipment was composed of an HPLC (model 1090), with a diode-array detector, commanded by an HPLC ChemStation. When the analysis was performed using TLC, 3 μl aliquots of the incubation mixture were spotted onto the TLC plates. Standards of Ap<sub>4</sub>A were applied at the origins and the plates were developed for 90 min in dioxane/ammonia/water (6:1:4, by vol.). Spots corresponding to Ap<sub>4</sub>A were cut out and the radioactivity counted. For estimation of the K<sub>m</sub>, pH optimum, substrate specificity and metal-ion requirements, the standard incubation mixture was appropriately modified.

## RESULTS

### Metal requirements

Previously [1], it was mentioned briefly that the synthesis of Ap<sub>4</sub>A, catalyzed by firefly luciferase, was strictly dependent on some divalent cations. We have characterized the dependence and Fig. 1 shows that maximum luciferase activity was obtained in the presence of 2.5–5 mM MgCl<sub>2</sub>. No activity was observed when exogenous metal was omitted and in the presence of an excess of EDTA (results not shown). Zn<sup>2+</sup>, Co<sup>2+</sup> and Mn<sup>2+</sup> could replace Mg<sup>2+</sup>, but the velocities were lower. Ca<sup>2+</sup> was even less effective: maximum velocity, obtained at 10 mM CaCl<sub>2</sub>, was less than 10% of that observed in the presence of 2.5 mM MgCl<sub>2</sub>. In a separate experiment using the same assay conditions as in Fig. 1, the synthesis of Ap<sub>4</sub>A was measured in the presence of different metal chlorides at 5 mM concentration. The relative velocities were as follows: Mg<sup>2+</sup>, 100; Zn<sup>2+</sup>, 54; Mn<sup>2+</sup>, 44; Ni<sup>2+</sup>, 8; Cd<sup>2+</sup>,

7;  $\text{Ca}^{2+}$ , 5;  $\text{Ba}^{2+}$ , <1;  $\text{Cu}^{2+}$ , <1.  $\text{Zn}^{2+}$ , which strongly stimulated the adenosine 5',5'''- $P^1, P^4$ -tetraphosphonucleoside ( $\text{Ap}_4\text{N}$ ) or adenosine 5',5'''- $P^1, P^3$ -triphosphonucleoside ( $\text{Ap}_3\text{N}$ ) synthesis, catalyzed by some aminoacyl-tRNA synthetase [11–15], did not affect the  $\text{Ap}_4\text{A}$  synthesis catalyzed by firefly luciferase when added to the standard incubation mixture.

#### Effect of pH

To estimate the optimum pH for  $\text{Ap}_4\text{A}$  synthesis, the incubation was carried out in 50 mM of each buffer as indicated in Fig. 2. The reaction presented maximal rates at pH values around 5.7. At more acidic pH values, the enzyme activity decreased sharply, being undetectable at pH 4. At more basic pH values, the activity decreased more slowly, still being 20% of the maximum velocity at pH 9 (Fig. 2).

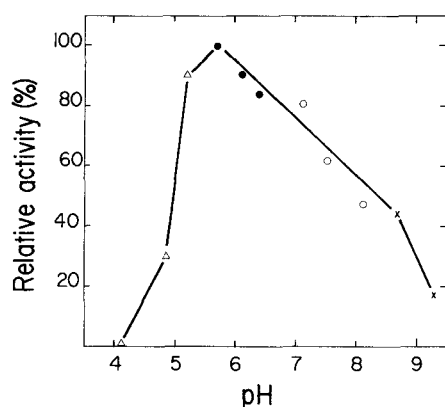


Fig. 2. Effect of pH on the initial rate of  $\text{Ap}_4\text{A}$  synthesis, catalyzed by firefly luciferase. The reaction mixture (25  $\mu\text{l}$ ) contained 50 mM buffer ( $\Delta$  acetate;  $\bullet$  Mes;  $\circ$  Hepes;  $\times$  Ches) as indicated, 0.25 mM luciferin, 2.5 mM [2,8- $^3\text{H}$ ] ATP (1  $\mu\text{Ci}$ ), 0.05 U inorganic pyrophosphatase and luciferase (25  $\mu\text{g}$  protein). To estimate initial rates, aliquots were withdrawn after 10, 20 and 40 min of incubation and analyzed by TLC

#### $K_m$ values

The  $K_m$  value for luciferin in the synthesis of  $\text{Ap}_4\text{A}$ , catalyzed by the firefly luciferase, was determined in the presence of 2 mM ATP and increasing amounts of luciferin (1, 2, 4, 12 and 40  $\mu\text{M}$ ). A control without luciferin was also run. Aliquots of the reaction mixtures were withdrawn after 20 min and 40 min of incubation and analyzed by HPLC. The  $K_m$  values calculated for both series of experiments were around 2–3  $\mu\text{M}$ . Slight inhibition of the  $\text{Ap}_4\text{A}$  synthesis was observed at higher (>0.3 mM) luciferin concentrations. Traces of  $\text{Ap}_4\text{A}$  synthesis were measured in the control mixture, which was not supplemented with exogenous luciferin, probable due to the presence of luciferin in the commercial luciferase preparations. Comparison of the rate of  $\text{Ap}_4\text{A}$  synthesis, observed in the absence of exogenous luciferin, with the rates obtained at different luciferin concentrations, enabled us to calculate that about 7 pmol endogenous luciferin were present in the added luciferase preparations (37  $\mu\text{g}$  protein). The  $K_m$  value for ATP, determined in the presence of 0.1 mM luciferin, was 4 mM (results not shown).

#### Substrate specificity

The substrate specificity of the enzyme, with respect to nucleotides, was tested in two different experiments, involving the synthesis of homo-dinucleoside polyphosphates (Table 1) or homo-dinucleoside and hetero-dinucleoside polyphosphates (Table 2). In both cases, samples were taken from the reaction mixtures after 0, 2 and 20 h of incubation and analyzed by HPLC. The syntheses of DNPPs obtained were used to measure the rate and the extent of the reaction.

From the rate of synthesis of homo-DNPPs, one can judge the efficiency of the assayed nucleotide, both as a nucleotide donor and a nucleotide acceptor. Synthesis was tested with the following nucleotides: ATP, adenosine 5'-tetrphosphate ( $\text{p}_4\text{A}$ ), dATP,  $\text{App}[\text{CH}_2]_p$ ,  $\text{Ap}[\text{CH}_2]_p$ , ( $\text{Sp}$ )-ATP[ $\alpha\text{S}$ ], ATP[ $\gamma\text{S}$ ] and GTP. As shown in Table 1, decreasing rates of synthesis of homodinucleoside polyphosphates [ $\text{Ap}_4\text{A}$  > diadenosine 5',5'''- $P^1, P^5$ -pentaphosphate ( $\text{Ap}_5\text{A}$ ) > dideoxyadenosine 5',5'''- $P^1, P^4$ -tetrphosphate ( $\text{dAp}_4\text{dA}$ ) >

Table 1. Synthesis of homodinucleoside polyphosphates catalyzed by luciferase

The reaction mixture (50  $\mu\text{l}$ ) contained luciferase (25  $\mu\text{g}$  protein), the nucleotides shown below and the rest of the components as described in Materials and Methods. At the indicated times, aliquots were withdrawn and analyzed by HPLC. Velocity ( $v$ ) was calculated from the values obtained after 2 h incubation. Figures indicate nmoles of the corresponding nucleotide present in the assay mixture

Nucleotide added	Dinucleotide synthesized	Nucleotide present at time			$v$
		0 h	2 h	20 h	
		nmol			nmol/min/mg protein
ATP	$\text{Ap}_4\text{A}$	86	26	1	—
			27	36	9
$\text{p}_4\text{A}$	$\text{Ap}_5\text{A}$	65	34	19	
			12	17	4
dATP	$\text{dAp}_4\text{dA}$	87	62	8	
			9	30	3
GTP	$\text{Gp}_4\text{G}$	97	91	69	
			2.4	10	0.8
ATP[ $\gamma\text{S}$ ]	$\text{Ap}_4\text{A}[\beta\text{S}]$	50	49	41	
			<0.1	3.2	

Table 2. Synthesis of homo-dinucleoside and hetero-dinucleoside polyphosphates catalyzed by luciferase

The reaction mixture (50  $\mu$ l) contained luciferase (25  $\mu$ g protein), the nucleotides shown below and the other components of the assay as in Table 1. Aliquots were withdrawn at the indicated times of incubation and analyzed by HPLC. Figures indicate nmoles of the corresponding nucleotide present in the assay mixture

Nucleotides added	Dinucleotides synthesized	Nucleotide present at time		
		0 h	2 h	20 h
nmol				
ATP		86	11	4
p <sub>4</sub> A		65	41	18
	Ap <sub>4</sub> A		17	
	Ap <sub>5</sub> A		29	47
ATP		86	24	4
dATP		87	50	5
	Ap <sub>4</sub> A		18	22
	Ap <sub>4</sub> dA		24	47
	dAp <sub>4</sub> dA		3	11
ATP		86	90	70
App[CH <sub>2</sub> ]p		80	80	70
	Ap <sub>4</sub> A		27	28
	App[CH <sub>2</sub> ]ppA		2	13
ATP		86	25	3
Ap[CH <sub>2</sub> ]pp		87	67	24
	Ap <sub>4</sub> A		30	13
	Ap[CH <sub>2</sub> ]pppA		16	57
ATP		100	39	
(Sp)ATP[ $\alpha$ S]		80	67	23
	Ap <sub>4</sub> A		26	18
	(Sp)Ap <sub>4</sub> A[ $\alpha$ S]		10	65
ATP		100	55	2
ATP[ $\gamma$ S]		68	68	46
	Ap <sub>4</sub> A		28	60
	Ap <sub>4</sub> A[ $\beta$ S]			3
ATP		86	12	15
GTP		100	67	24
	Ap <sub>4</sub> A		22	9
	Ap <sub>4</sub> G		33	64
ATP		86	36	2
ADP		54	54	47
	Ap <sub>4</sub> A		29	42
	Ap <sub>3</sub> A			1

Gp<sub>4</sub>G] were measured, after a 2-h incubation, using ATP, p<sub>4</sub>A, dATP and GTP, respectively, as substrates. No detectable synthesis of dinucleotides was observed with the other nucleotides tested. After 20 h of incubation, almost complete conversion of ATP and dATP into Ap<sub>4</sub>A and dAp<sub>4</sub>dA, respectively, was observed; less effective synthesis of the corresponding dinucleotides was observed with p<sub>4</sub>A, GTP and ATP[ $\gamma$ S] and, finally, in the reaction mixtures containing either App[CH<sub>2</sub>]p, Ap[CH<sub>2</sub>]pp or (Sp)-ATP[ $\alpha$ S] no appreciable synthesis of dinucleotides was measured.

The failure to achieve synthesis of homo-DNPPs with some nucleotide 5'-triphosphates can be interpreted as being due either to their inability to form the E-LH<sub>2</sub>-NMP complex or their inability to transfer the nucleotidyl moiety of the complex to another NTP molecule. To further investigate this

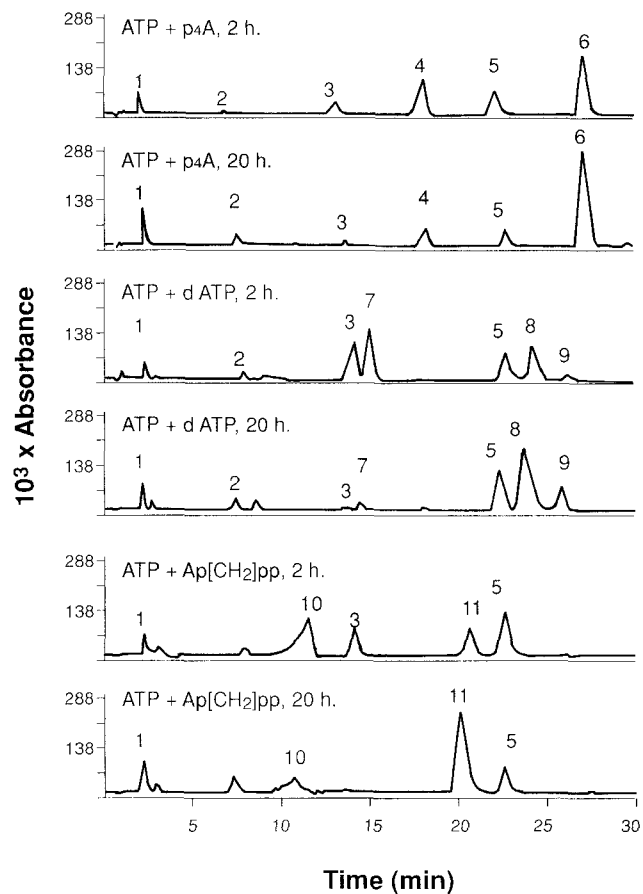


Fig. 3. Synthesis of homo-dinucleotides and hetero-dinucleotides by firefly luciferase. The reaction mixture contained the indicated nucleotides at 2 mM final concentration, luciferase and the other components as described in Materials and Methods. After 2 h and 20 h incubation, 15  $\mu$ l were withdrawn and analyzed by HPLC. Absorbance was measured at 260 nm. The figures at the top of the chromatographic peaks correspond to the following nucleotides: (1) AMP; (2) ADP; (3) ATP; (4) p<sub>4</sub>A; (5) Ap<sub>4</sub>A; (6) Ap<sub>5</sub>A (7) dATP; (8) Ap<sub>4</sub>dA; (9) dAp<sub>4</sub>dA; (10) Ap[CH<sub>2</sub>]pp; (11) Appp[CH<sub>2</sub>]pA

problem, the ability of the enzyme to transfer the adenylyl moiety of the E-LH<sub>2</sub>-AMP complex to different nucleotides was tested. In this case, the incubation mixture contained, in addition to ATP, one of the previously tested nucleotides (see above). From the results obtained after 2 h of incubation (Table 2 and Fig. 3), it appeared that the rate of Ap<sub>4</sub>A synthesis was lower than that of the synthesis of either Ap<sub>5</sub>A, adenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetraphosphodeoxyadenosine (Ap<sub>4</sub>dA) or adenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetraphosphoguanosine (Ap<sub>4</sub>G) when the reaction mixture contained (in addition to ATP) p<sub>4</sub>A, dATP or GTP, respectively. The rate of synthesis of diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>[ $\alpha,\beta$ -methylene]tetraphosphate (Ap[CH<sub>2</sub>]pppA), diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>[ $\beta,\gamma$ -methylene]tetraphosphate (App[CH<sub>2</sub>]ppA), and (Sp)-diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[ $\alpha$ -thio]tetraphosphate ((Sp)-Ap<sub>4</sub>A[ $\alpha$ S]) was lower than that of Ap<sub>4</sub>A. In the mixtures containing ATP and ATP[ $\gamma$ S] or ADP, only traces of diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-[ $\beta$ -thio]tetraphosphate (Ap<sub>4</sub>A[ $\beta$ S]; 3 nmol) or diadenosine triphosphate (Ap<sub>3</sub>A; 1 nmol) were detected after 20 h incubation. The results presented in Table 2 show that, whereas the amount of synthesized hetero-DNPPs increased markedly when the incubation was prolonged over 2–20 h, the amount of the initially

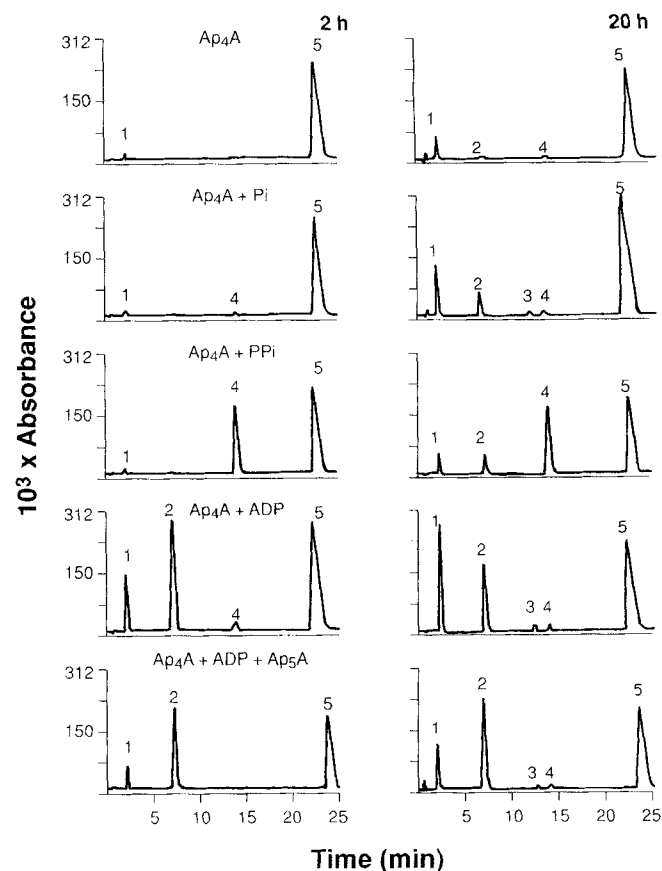
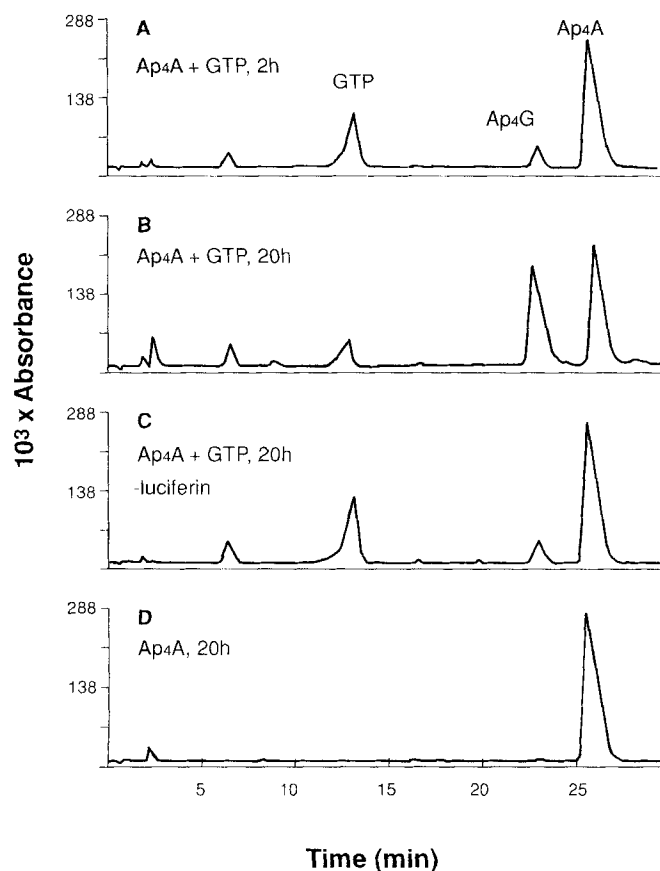


Fig. 4. Splitting of  $Ap_4A$  by luciferase in the presence of GTP. The reaction mixture contained  $Ap_4A$  and GTP at 2 mM final concentration, luciferase and the other components as described in Material and Methods. After 2 h and 20 h incubations, 15  $\mu$ l were withdrawn and analyzed by HPLC (A, B). Controls were run in the absence of luciferin (C) or in the absence of GTP (D). Absorbance was at 260 nm

Fig. 5. Splitting of  $Ap_4A$  by luciferase in the presence of  $P_i$  or  $PP_i$  or ADP. The reaction mixture contained  $Ap_4A$  and  $P_i$  or  $PP_i$  or ADP at 2 mM final concentration. The rest of the components were as described in Materials and Methods, except that pyrophosphatase was omitted when  $PP_i$  was present. Where indicated, 0.05 mM  $Ap_5A$  was added in addition to  $Ap_4A$  and ADP. After 2 h and 20 h incubations, (left and right of the figure, respectively), 15  $\mu$ l were withdrawn and analyzed by HPLC. A control (top chromatograms) was also run to estimate the splitting of  $Ap_4A$  by luciferase. Absorbance was at 260 nm. The figures at the top of the chromatographic peaks correspond to the following nucleotides: (1) AMP; (2) ADP, (3)  $Ap_3A$ ; (4) ATP; (5)  $Ap_4A$

synthesized  $Ap_4A$  decreased in many cases. This trend was particularly striking in the reaction mixtures containing ATP and GTP. Note that the amount of ATP still present in the reaction mixture was almost the same after 2 h and 20 h incubation, whereas the amount of  $Ap_4G$  almost doubled during that period.

*The splitting of  $Ap_4A$  by luciferase is accelerated in the presence of nucleoside triphosphates and  $PP_i$*

As deduced from the results of Table 2, the commercial luciferase preparation seemed to be able to split  $Ap_4A$ . To further explore this possibility,  $Ap_4A$  (2 mM) was incubated in the presence of 2 mM GTP and the nucleotide content of the mixture analyzed after 2 h and 20 h (Fig. 4A, B). Controls were carried out in the presence of GTP but absence of luciferin (Fig. 4C) and in the presence of luciferin but absence of GTP (Fig. 4D). It is clear that net synthesis of  $Ap_4G$  took place in the presence of  $Ap_4A$  and GTP (Fig. 4A, B) and that practically no splitting of  $Ap_4A$ , and hence no synthesis of  $Ap_4G$ , proceeded in the absence of luciferin (Fig. 4C) or GTP (Fig. 4D). The degradation of  $Ap_4A$  did not seem to be due to a contaminant phosphodiesterase or to  $Ap_4A$ -degrading enzymes present in the commercial preparation of luciferase, but rather to an inherent property of luciferase, which became

apparent when the enzyme was simultaneously challenged with  $Ap_4A$  and a NTP.

The splitting of  $Ap_4A$  by luciferase was also measured after 2 h and 20 h incubation, in the presence of  $P_i$ ,  $PP_i$  or ADP (Fig. 5). With  $P_i$  in the reaction mixture, small amounts of AMP, ADP,  $Ap_3A$  and ATP were observed only after 20 h of incubation. In the presence of  $PP_i$ ,  $Ap_4A$  gave rise to ATP at a higher rate (fivefold) than that of  $Ap_4G$  formation when the splitting of  $Ap_4A$  by luciferase was carried out in the presence of GTP (results not shown). In the presence of ADP (Fig. 5), a peak corresponding to  $Ap_3A$  appeared after 20 h but not after 2 h of incubation. The high amount of AMP at the longer incubation time suggested the presence of contaminant adenylate kinase in the commercial luciferase. This was actually the case, as shown by the formation of  $Ap_4A$ , AMP and traces of ATP when luciferase was incubated with ADP (results not shown).  $Ap_4A$  was, in this case, synthesized by luciferase from the ATP generated by the kinase. To partially avoid the effect of adenylate kinase, the splitting of  $Ap_4A$  by luciferase in the presence of ADP was studied adding  $Ap_5A$

(a known inhibitor [23] of adenylate kinase) to the reaction mixture. Under these conditions, the amount of AMP produced after 20 h incubation is clearly lower than in the absence of Ap<sub>5</sub>A (Fig. 5).

## DISCUSSION

Although common mechanistic features in the reactions catalyzed by fatty acyl-CoA synthetases, aminoacyl-tRNA synthetases and firefly luciferase were highlighted many years ago [19], until very recently the ability to produce adenylated nucleotides (Ap<sub>4</sub>N and Ap<sub>3</sub>N) had been recognized only for some aminoacyl-tRNA synthetases [10–16]. We have recently demonstrated [1], however, that commercially available preparations of firefly luciferase easily form Ap<sub>4</sub>A when the incubation mixture contains ATP, luciferin and pyrophosphatase. In this study, we have characterized that reaction in more detail.

Like most enzymes involved in nucleotide metabolism, luciferase requires Mg<sup>2+</sup> as the preferred divalent cation. Interestingly, Zn<sup>2+</sup> could substitute for Mg<sup>2+</sup> and sustain Ap<sub>4</sub>A synthesis at a rate only twofold lower. In contrast to the effect exerted on the phenylalanyl-tRNA synthetase, lysyl-tRNA synthetase and alanyl-tRNA synthetase [11–15], Zn<sup>2+</sup> did not act synergistically when added to the luciferase mixtures containing Mg<sup>2+</sup>.

With respect to the pH optimum (5.7) of the Ap<sub>4</sub>A synthesis, the enzyme resembles the yeast Ap<sub>4</sub>A phosphorylase, which catalyzes the synthesis of Ap<sub>4</sub>A from ATP and ADP most effectively at pH 5.9 [24].

From the experiments presented in Tables 1 and 2, the following properties on the nucleotide specificity of the luciferase can be deduced.

Although we have not directly measured the formation of the E-LH<sub>2</sub>-NMP complex, it can be inferred that adenine nucleotides (ATP > p<sub>4</sub>A > dATP) are the preferred substrates for the synthesis of this complex. In the presence of GTP, the rate of synthesis of Gp<sub>4</sub>G was about 10% of that obtained for the synthesis of Ap<sub>4</sub>A from ATP. Even less synthesis of Ap<sub>4</sub>A[βS] was obtained from ATP[γS] and undetectable amounts of the corresponding homo-dinucleoside polyphosphates were obtained in the presence of App[CH<sub>2</sub>]<sub>2</sub>p, Ap[CH<sub>2</sub>]<sub>2</sub>pp or (Sp)ATP[αS].

From the data shown in Table 2, it appears that the transfer of the adenylyl residue from the E-LH<sub>2</sub>-AMP complex to various acceptors (ATP, p<sub>4</sub>A, dATP, GTP and Ap[CH<sub>2</sub>]<sub>2</sub>pp) which have at least a three-phosphate chain and unmodified terminal pyrophosphate moiety, proceeds at comparable high rates. Nucleotides with only two phosphates (ADP) or with a modified terminal pyrophosphate (App[CH<sub>2</sub>]<sub>2</sub>p and ATP[γS]) much less efficiently support the synthesis of the corresponding dinucleotides.

Concerning the synthesis of phosphorothioate analogues of Ap<sub>4</sub>A from (Sp)ATP[αS] and ATP[γS], the results obtained are in line with those discussed above, i.e. the luciferase accepted ATP[αS] as an adenylate acceptor quite well, but not as an adenylate donor; in contrast, ATP[γS] was a poor adenylate acceptor, but behaved as a good adenylate donor, since in reaction mixtures containing equimolar amounts of ATP[γS] and GTP, complete conversion of both nucleotides to Ap<sub>4</sub>G was obtained (results not shown).

In the presence of Ap<sub>4</sub>A and a nucleoside triphosphate (NTP), luciferase is able to split Ap<sub>4</sub>A, giving Ap<sub>4</sub>N. The amount of Ap<sub>4</sub>N synthesized reflects the utilization of the two AMP moieties of Ap<sub>4</sub>A (results not shown), which suggests

that Ap<sub>4</sub>A cleavage proceeds through formation of ATP and E-LH<sub>2</sub>-AMP. Subsequent synthesis of a second E-LH<sub>2</sub>-AMP from ATP allows the transfer of 2 mol AMP from Ap<sub>4</sub>A to 2 mol NTP.

The results presented in Fig. 5 can be interpreted on the basis that, once the E-LH<sub>2</sub>-AMP complex is formed in the presence of Ap<sub>4</sub>A, its adenylyl moiety is readily accepted by PP<sub>i</sub> producing ATP by reversion of reaction (1). Only traces of ADP and Ap<sub>3</sub>A are synthesized when Ap<sub>4</sub>A is incubated in the presence of P<sub>i</sub> or ADP, indicating that the adenylyl moiety of the complex is very poorly accepted by these two compounds.

There are similarities and differences concerning the synthesis of dinucleoside polyphosphates by aminoacyl-tRNA synthetases, Ap<sub>4</sub>A phosphorylase and luciferase. With respect to the structure of the adenylate acceptor, the luciferase requirements are similar to those of the Ap<sub>4</sub>A phosphorylase. The latter enzyme transfers adenylate (nucleotide) presumably from a covalent enzyme-nucleotide complex to those purine ribonucleotides which contain at least a three-phosphate chain at the 5'-position of the nucleotide residue; i.e. to ATP, GTP or p<sub>4</sub>A, but not to NDP [22, 24]. Both luciferase and lysyl-tRNA synthetase from *E. coli* [9] accept (Sp)ATP[αS] as an adenylate acceptor, but not as an adenylate donor. The inability to use NDPs as adenylate acceptors distinguishes firefly luciferase from the aminoacyl-tRNA synthetases which can synthesize both Ap<sub>4</sub>N and Ap<sub>3</sub>N at comparable rates [10, 12, 15]. There is also another difference between the Ap<sub>4</sub>A synthesis catalyzed by firefly luciferase and aminoacyl-tRNA synthetases. Under standard conditions firefly luciferase, converts ATP almost completely into Ap<sub>4</sub>A (Table 1), whereas the aminoacyl-tRNA synthetases convert Ap<sub>4</sub>A, which accumulates in the early stages of incubation, into ADP and/or Ap<sub>3</sub>A [11, 13, 16].

Since p<sub>4</sub>A and guanosine 5'-tetrphosphate (p<sub>4</sub>G) (for the latter, results not shown) appeared to be good adenylate acceptors, it is plausible that, at least in the cells of the firefly lanterns, the dinucleoside pentaphosphates, Ap<sub>5</sub>A and adenosine 5',5'''-P<sup>1</sup>,P<sup>2</sup>-pentaphosphoguanosine (Ap<sub>5</sub>G), may occur. The presence of those (or others) pentaphosphates have not yet been reported for any tissue.

Firefly luciferase is widely used for the estimation of dinucleoside tetraphosphates [17, 20, 21]. In those assays, the hydrolysis of dinucleotides by snake venom phosphodiesterase is coupled to the luciferase either directly (in the case of Ap<sub>4</sub>N) [17, 20, 21] or indirectly [in the case of dinucleoside 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetraphosphates (Np<sub>4</sub>N)] [17, 21] through other coupled enzymatic reactions that produce ATP. The possible artifacts due to the synthesis of Ap<sub>4</sub>A are, in our view, negligible, because of both the absence of pyrophosphatase in those assays and the big difference in the K<sub>m</sub> for ATP, which are in the micromolar range for the formation of the E-LH<sub>2</sub>-AMP complex [19] (and hence light emission) and in the millimolar range for the synthesis of Ap<sub>4</sub>A. It is also worthy mentioning that Momsen (1978) already noticed that luciferase produced light with p<sub>4</sub>A and Ap<sub>5</sub>A with an intensity 2.2% and 0.75%, respectively, relative to ATP [25].

Novel properties of the firefly luciferase described in this paper can be useful for effective DNPP-synthase. One has to be aware, however, that the enzyme can not produce dinucleoside 5',5'''-P<sup>1</sup>,P<sup>3</sup>-triphosphates (Np<sub>3</sub>N), although the feature might, in some circumstances, be seen as an advantage.

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