

# Time course of luciferyl adenylate synthesis in the firefly luciferase reaction

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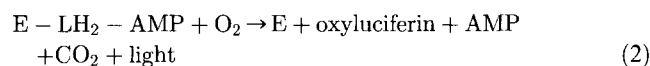
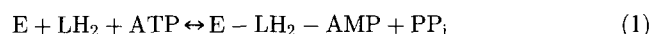
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**Abstract** The time course of luciferyl adenylate formation in the reaction catalyzed by firefly luciferase (EC 1.13.12.7) has been followed. The properties of luciferyl adenylate, enzymatically or chemically synthesized, as substrate of luciferase, have been compared. The potential use of luciferyl adenylate for luciferase detection is here proposed.

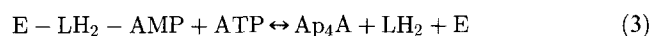
**Key words:** Firefly luciferase; Luciferin; ATP; Luciferyl adenylate; Enzyme kinetics

## 1. Introduction

Data in the literature support the contention that a luciferyl adenylate intermediate complex is formed during the luciferase-catalyzed reaction although its enzymatic synthesis has not been clearly demonstrated nor has its time course of formation been followed [1–6]. The proposed scheme of the reaction is the following:



Recently it was shown that luciferase, in the absence or presence of oxygen, catalyzes the synthesis of  $Ap_4A$  using ATP as substrate [7,8]. Dehydroluciferin can substitute for luciferin in this reaction [9], which is greatly accelerated in the presence of pyrophosphatase [7].



While studying the mechanism of synthesis of  $Ap_4A$ , we discovered, accidentally, the experimental conditions under which the synthesis and utilization of the luciferyl adenylate intermediate complex could be observed.

## 2. Materials and methods

### 2.1. Materials

Luciferase from firefly (*Photinus pyralis*) was purchased from Sigma (catalogue no. L-5226). A stock solution of luciferase was prepared by dissolving 5 mg of powder in 1 ml of 0.5 M HEPES/KOH (pH 7.5). Inorganic pyrophosphatase (catalogue no. 108987) and phosphodiesterase from *Crotalus Durissus* (catalogue no. 108260) were obtained

from Boehringer. D-Luciferin, ATP and AMP were purchased from Sigma. Toyopearl HW-40 gel was from Toyo Soda MFG, Japan and Micropure 0.45 separators were from Amicon (catalogue no. 42522).

Bioluminescence was measured in a luminometer (Lumat LB 9501) from Berthold, integrating the light signal over an initial period 3 s. In all experiments mean values of at least two determinations are given. Fluorescence was measured in a Perkin Elmer Luminescence Spectrometer (LS 50).

### 2.2. Enzymatic synthesis and purification of luciferyl adenylate ( $LH_2$ -AMP)

The reaction mixture contained, in a final volume of 0.05 ml: 50 mM HEPES/KOH (pH 7.5), 5 mM  $MgCl_2$ , 0.5 mg/ml luciferase, 2 mM ATP, 0.1 mM D-luciferin, and 5  $\mu$ g/ml inorganic pyrophosphatase. After 15 min of incubation the reaction was stopped by adding 1 N HCl to a pH of 3.5, and then heated for 1.5 min in boiling water. After cooling, the mixture was filtered by centrifugation through a Micropure 0.45 separator to remove denatured proteins. The filtrate was applied to a small syringe containing 1.5 ml of Toyopearl HW 40 gel, equilibrated with water (pH 3.5) and fractions of 0.65 ml were collected (Fig. 1). Concentrations of ATP and  $LH_2$  in the effluent were determined by bioluminescence using an internal standard. The chromatographic profile of  $LH_2$ -AMP is presented in relative light units (RLU). Chemically synthesized  $LH_2$ -AMP eluted in the same fractions (results not shown).

### 2.3. Chemical synthesis of $LH_2$ -AMP

$LH_2$ -AMP was obtained as described in [1]. 2 mg of solid crystalline D-luciferin were mixed with 4 mg of crystalline solid sodium adenylate. To this mixture 4 ml of pyridine containing 400 mg of dicyclohexylcarbodiimide were added. The mixture was incubated at room temperature for about 1 h with occasional shaking, after which 20 ml of 50 mM bicarbonate buffer (pH 7.8) was added. The cloudy mixture was clarified by three successive extractions with a total volume of 60 ml of ether. The watery solution was adjusted to pH 2.5 with 1 N HCl and extracted three times with a total volume of 60 ml of redistilled ethyl acetate. Traces of ethyl acetate were removed by passing a stream of helium through the mixture. The solution was adjusted to pH 3.5 with KOH and kept at  $-20^\circ\text{C}$ . Under those conditions no detectable hydrolysis was observed after 1 month of storage.

## 3. Results and discussion

### 3.1. Time course of the enzymatic synthesis of $LH_2$ -AMP

In the course of an investigation aimed to measure the initial rate of disappearance of ATP in the reaction mixture used to synthesize  $Ap_4A$ , we unexpectedly detected the synthesis of one compound, different from ATP, and substrate of luciferase. A typical result is presented in Fig. 2. The reaction mixture contained, among other reactants (see Section 2), luciferase, 0.1 mM luciferin and 2 mM ATP. Controls without ATP, luciferin, pyrophosphatase or luciferase were run in parallel. At several time points of incubation aliquots were taken, diluted 100-fold in water (pH 3.5), kept for 2 h at room temperature and the bioluminescence of 0.01 ml aliquots determined. As shown in Fig. 2, in the complete reaction mixture the bioluminescence signal increased sharply, attaining a maximum at around 10 min of incubation, and

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**Abbreviations:**  $Ap_4A$ , diadenosine 5',5''- $P^1$ , $P^4$ -tetrphosphate or diadenosine tetrphosphate; E, luciferase;  $LH_2$ , D-luciferin;  $LH_2$ -AMP, luciferyl adenylate; RLU, relative light units

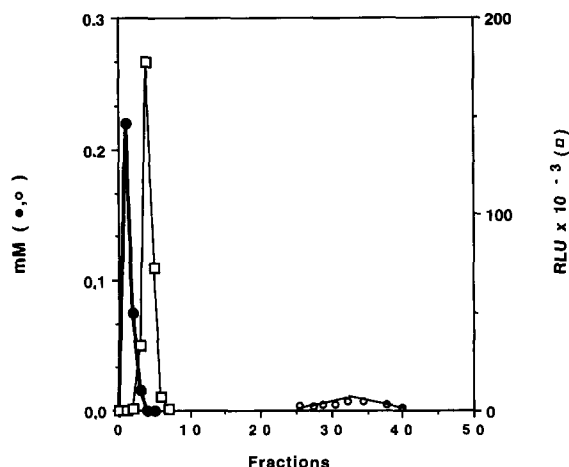


Fig. 1. Purification of luciferyl-AMP.  $\text{LH}_2$ -AMP was synthesized enzymatically and treated as described in Section 2. The reaction mixture was applied to a 1.5 ml Toyopearl HW 40 column, equilibrated and eluted with water (pH 3.5); fractions of 0.65 ml were collected. ATP (●), luciferin (○) and  $\text{LH}_2$ -AMP (□), were determined by bioluminescence.

decreased slowly thereafter. The intensity of the light emitted at 10 min of incubation is around 50-times higher than the corresponding control treated in the same way, in which luciferase was omitted (Fig. 2). In the absence of pyrophosphatase the luminescence signal is less intense and peaks earlier.

### 3.2. Comparative properties of enzymatically and chemically synthesized $\text{LH}_2$ -AMP

As a working hypothesis we assumed that the substrate of luciferase responsible for the unexpected luminescence signal (Fig. 2) could be  $\text{LH}_2$ -AMP. To check this hypothesis, the presumptive enzymatically synthesized  $\text{LH}_2$ -AMP was purified (see Section 2 (Fig. 1)),  $\text{LH}_2$ -AMP was chemically synthesized, and the properties of both compounds compared. The fluorescence excitation and emission spectra of both  $\text{LH}_2$ -AMPs at pH 3.5 were the same as for luciferin. Taking into account that the fluorescence intensity of  $\text{LH}_2$ -AMP is about 0.45 that of luciferin [10], we calculated that the concentration of the presumptive  $\text{LH}_2$ -AMP formed enzymatically (Fig. 2) reached a maximum concentration of around 100 nM. The calculated  $K_m$  value found for the presumptive  $\text{LH}_2$ -AMP, measuring the light intensity under conditions when the enzyme concentration was higher than the  $\text{LH}_2$ -AMP concen-

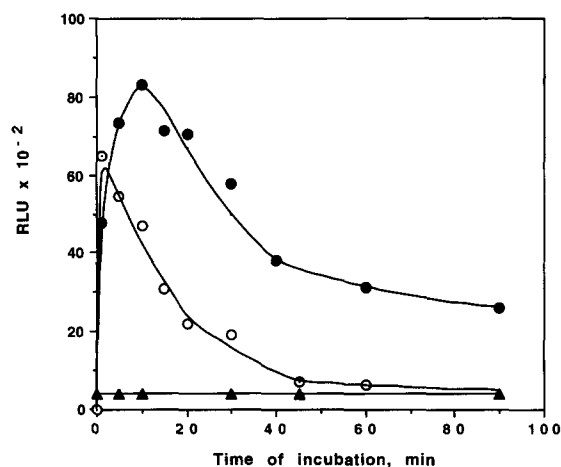


Fig. 2. Time course of the synthesis and utilisation of luciferyl adenylate in the luciferase reaction. The reaction mixture contained, in a final volume of 0.05 ml: 50 mM HEPES/KOH (pH 7.5), 5 mM  $\text{MgCl}_2$ , 0.5 mg/ml luciferase, 2 mM ATP, 0.1 mM luciferin, 5  $\mu\text{g/ml}$  inorganic pyrophosphatase. Incubation was performed at room temperature. At the times indicated 5- $\mu\text{l}$  aliquots were withdrawn and diluted in 0.495 ml of distilled water adjusted to pH 3.5 with HCl. After 2 h at room temperature, 0.01-ml aliquots were added to 0.49 ml of a reaction mixture containing 50 mM HEPES/KOH (pH 7.5), 10 mM  $\text{MgCl}_2$  and 0.4  $\mu\text{g/ml}$  luciferase, and bioluminescence determined. Complete assay (●); omitting pyrophosphatase (○), or omitting luciferase (▲).

tration [11], was around  $1.7 \times 10^{-7}$  M, similar to the value obtained previously ( $2.3 \times 10^{-7}$  M) [1] using chemically synthesized  $\text{LH}_2$ -AMP.

Both compounds were also compared as substrates of luciferase in the presence of added ATP, or luciferin, or in the absence of  $\text{Mg}^{2+}$  (Table 1). The results obtained with presumptive  $\text{LH}_2$ -AMP were essentially the same as those with

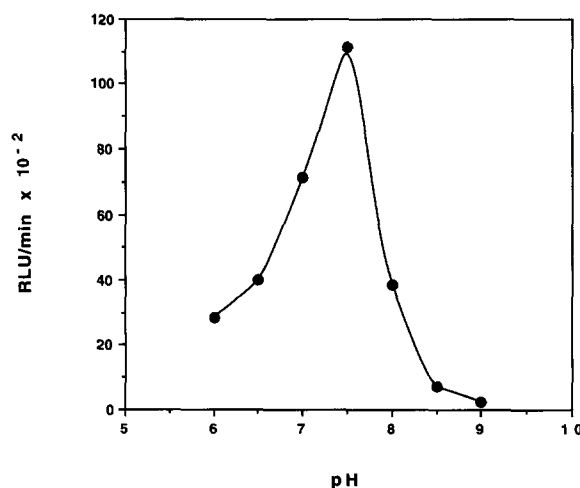


Fig. 3. Influence of pH on the luciferase-dependent synthesis of luciferyl adenylate. The reaction mixture was as in Fig. 2 except that 0.8 M HEPES-KOH buffer was used at the pH values indicated and the amount of luciferase decreased to 0.1 mg/ml. At different times of incubation aliquots were taken, diluted and the luminescence activity determined as described under Fig. 2. For each pH, a plot similar to that in Fig. 2 was obtained, the initial velocities determined and represented as a function of the pH value in the reaction mixture.

Table 1  
 $\text{LH}_2$ -AMP as substrate of luciferase

Reactants	$\text{LH}_2$ -AMP synthesized	
	Enzymatically	Chemically
E+ $\text{LH}_2$ -AMP+Mg	100	100
E+ $\text{LH}_2$ -AMP	63	64
E+ $\text{LH}_2$ -AMP+Mg+ATP	57	53
E+ $\text{LH}_2$ -AMP+Mg+ $\text{LH}_2$	23	27

The reaction mixture contained in a final volume of 0.5 ml: 50 mM HEPES/KOH (pH 7.5), 0.4  $\mu\text{g/ml}$  luciferase,  $\text{LH}_2$ -AMP (approx. 0.02 nM), and 10 mM  $\text{MgCl}_2$ . When ATP or luciferin were added, the final concentrations of those compounds were 1 mM and 2  $\mu\text{M}$ , respectively.

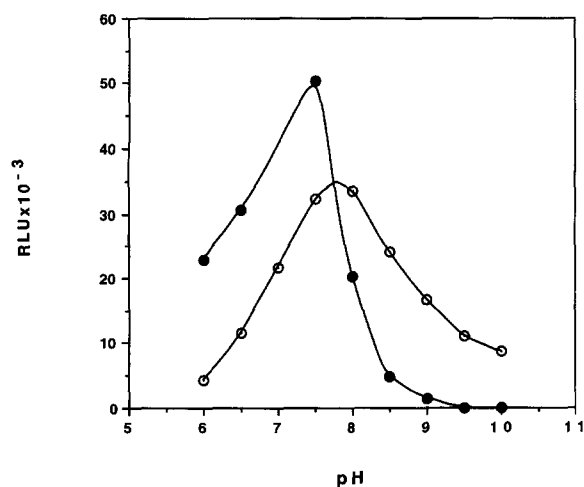


Fig. 4. pH dependence of bioluminescence reaction using ATP and luciferin, or presumptive luciferyl adenylate as substrates. The reaction mixture contained in a final volume of 0.5 ml: 50 mM HEPES/KOH (different pH values), 10 mM  $\text{MgCl}_2$ , 0.4  $\mu\text{g/ml}$  luciferase, and 20 nM luciferin plus 0.2 mM ATP (●), or a 0.01 ml aliquot of a 100-fold diluted sample withdrawn from a reaction mixture set up to synthesize presumptive  $\text{LH}_2$ -AMP as described in Fig. 2 (○). Luminescence was determined immediately after addition of the corresponding substrates.

$\text{LH}_2$ -AMP obtained by chemical synthesis, and in good agreement with previous results [1].

$\text{LH}_2$ -AMP from both sources was completely degraded after treatment with 4  $\mu\text{g/ml}$  phosphodiesterase for 30 min.

### 3.3. Influence of pH on the luciferase-dependent synthesis of luciferyl adenylate

The enzymatic formation of  $\text{LH}_2$ -AMP was followed at different pH values (Fig. 3). The reaction mixtures were adjusted to the pH values indicated in the figure. Aliquots were taken at different times of incubation and treated as described in Fig. 2. For each pH value a profile similar to that shown in Fig. 2 was obtained, the initial velocities of  $\text{LH}_2$ -AMP formation being determined and plotted as a function of pH in Fig. 3. Maximal activity was reached at a pH value around 7.8. Activity decreased sharply at pH values above and below this pH.

### 3.4. Influence of pH on the bioluminescence reaction using ATP and luciferin, or presumptive luciferyl adenylate as substrates

The pH dependence of luciferase was compared using ATP and luciferin, or presumptive  $\text{LH}_2$ -AMP as substrates of the reaction. Light emission, determined as described under Fig. 4, was maximal with both substrates at a pH value around 7.5–8.0, in accordance with previous results [1,12].

Taken together, these experiments demonstrate that under definite experimental conditions (2 mM ATP, 0.1 mM luciferin, 0.005 mM luciferase), it is possible to follow luciferyl adenylate formation during the luciferase-catalyzed reaction. This finding allows study of the influence of different chemical and physical factors on the process of its synthesis. Under our assay conditions (Fig. 2),  $\text{LH}_2$ -AMP at around 100 nM was at least 50-times more active in light production as compared with a control experiment where the concentrations of ATP and  $\text{LH}_2$  were around 4 and 3 orders of magnitude higher, respectively. Since luciferyl adenylate is rather stable at low pH values, it could be of potential use as a substrate to detect luciferase.

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