FEBS Letters 395 (1996) 188–190 FEBS 17644

Time course of luciferyl adenylate synthesis in the firefly luciferase reaction

Alexey Dukhovich, Antonio Sillero, Maria A. Günther Sillero*

Departamento de Bioquímica, Instituto de Investigaciones Biomedicas del CSIC, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain

Received 30 August 1996

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:

$$E + LH_2 + ATP \leftrightarrow E - LH_2 - AMP + PP_i \quad (1)$$

$$E - LH_2 - AMP + O_2 \rightarrow E + \text{oxyluciferin} + AMP + CO_2 + \text{light} \quad (2)$$

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].

$$E - LH_2 - AMP + ATP \leftrightarrow Ap_4A + LH_2 + E \quad (3)$$

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 μg/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°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

*Corresponding author. Fax: (34) (1) 397 53 53.

Abbreviations: Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate or diadenosine tetraphosphate; E, luciferase; LH_2, D-luciferin; LH_2-AMP, luciferyl adenylate; RLU, relative light units

PII S0014-5793(96)01038-1

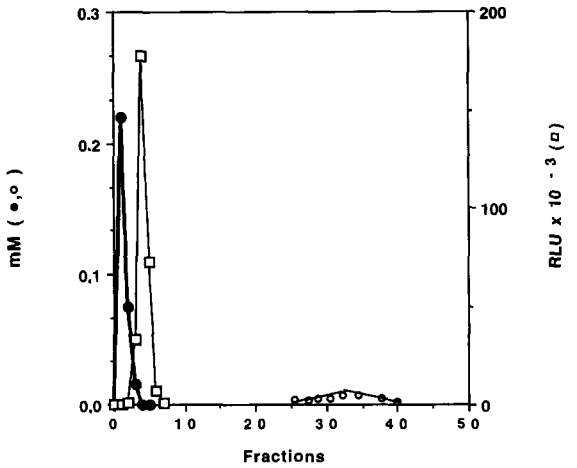

Fig. 1. Purification of luciferyl-AMP. LH_2-AMP was synthesized enzymatically and treated as described in Section 2. The reaction mixture was applied to a 1.5 ml Toyopearl HW 40 column, equilibrated and eluted with water (pH 3.5); fractions of 0.65 ml were collected. ATP (●), luciferin (○) and LH_2-AMP (□), were determined by bioluminescence.

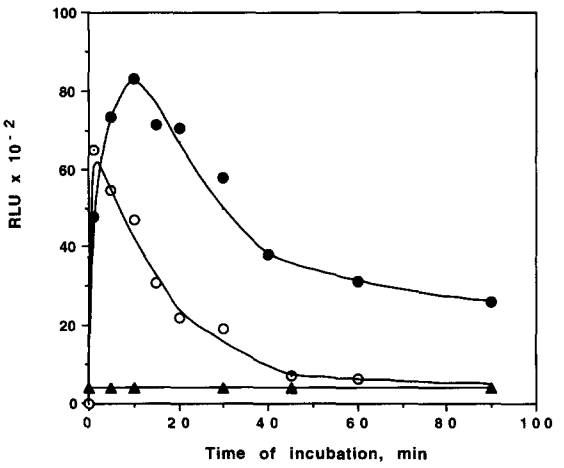

Fig. 2. Time course of the synthesis and utilisation of luciferyl adenylate in the luciferase reaction. The reaction mixture contained, in a final volume of 0.05 ml: 50 mM HEPES/KOH (pH 7.5), 5 mM $MgCl_2$, 0.5 mg/ml luciferase, 2 mM ATP, 0.1 mM luciferin, 5 μg/ml inorganic pyrophosphatase. Incubation was performed at room temperature. At the times indicated 5-μl 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 $MgCl_2$ and 0.4 μg/ml luciferase, and bioluminescence determined. Complete assay (●); omitting pyrophosphatase (○), or omitting luciferase (▲).

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 LH_2-AMP

As a working hypothesis we assumed that the substrate of luciferase responsible for the unexpected luminescence signal (Fig. 2) could be LH_2-AMP. To check this hypothesis, the presumptive enzymatically synthesized LH_2-AMP was purified (see Section 2 (Fig. 1)), LH_2-AMP was chemically synthesized, and the properties of both compounds compared. The fluorescence excitation and emission spectra of both LH_2-AMPs at pH 3.5 were the same as for luciferin. Taking into account that the fluorescence intensity of LH_2-AMP is about 0.45 that of luciferin [10], we calculated that the concentration of the presumptive LH_2-AMP formed enzymatically (Fig. 2) reached a maximum concentration of around 100 nM. The calculated K_m value found for the presumptive LH_2-AMP, measuring the light intensity under conditions when the enzyme concentration was higher than the LH_2-AMP concentration [11], was around 1.7×10^{-7} M, similar to the value obtained previously (2.3×10^{-7} M) [1] using chemically synthesized 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 Mg^{2+} (Table 1). The results obtained with presumptive LH_2-AMP were essentially the same as those with

Table 1
LH_2-AMP as substrate of luciferase

Reactants	LH_2-AMP synthesized	
	Enzymatically	Chemically
E+LH_2-AMP+Mg	100	100
E+LH_2-AMP	63	64
E+LH_2-AMP+Mg+ATP	57	53
E+LH_2-AMP+Mg+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 μg/ml luciferase, LH_2-AMP (approx. 0.02 nM), and 10 mM $MgCl_2$. When ATP or luciferin were added, the final concentrations of those compounds were 1 mM and 2 μM, respectively.

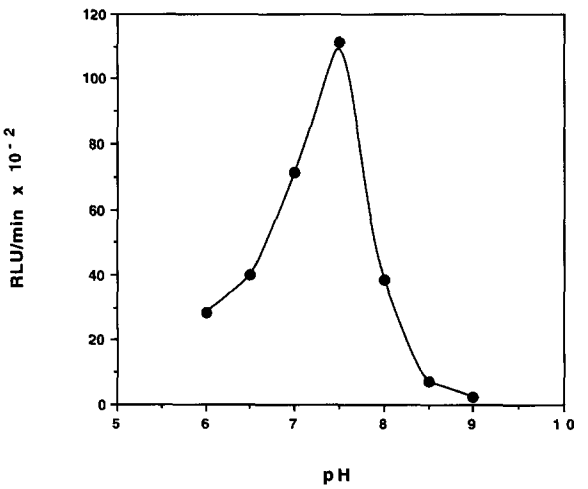

Fig. 3. Influence of pH on the luciferase-dependent synthesis of luciferyl adenylate. The reaction mixture was as in Fig. 2 except that 0.8 M HEPES-KOH buffer was used at the pH values indicated and the amount of luciferase decreased to 0.1 mg/ml. At different times of incubation aliquots were taken, 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.

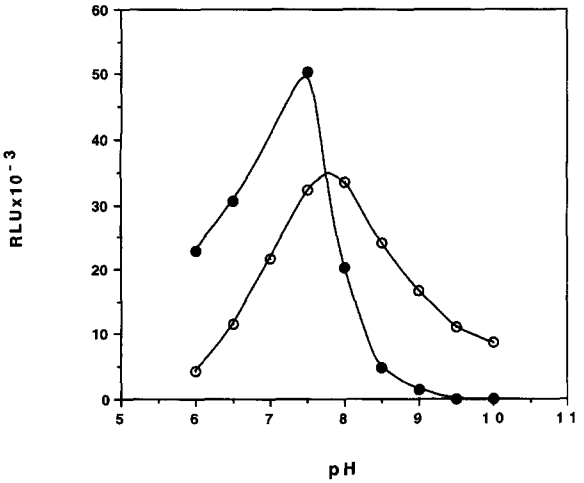

Fig. 4. pH dependence of bioluminescence reaction using ATP and luciferin, or presumptive luciferyl adenylate as substrates. The reaction mixture contained in a final volume of 0.5 ml: 50 mM HEPES/KOH (different pH values), 10 mM $MgCl_2$, 0.4 μg/ml luciferase, and 20 nM luciferin plus 0.2 mM ATP (●), or a 0.01 ml aliquot of a 100-fold diluted sample withdrawn from a reaction mixture set up to synthesize presumptive LH_2-AMP as described in Fig. 2 (○). Luminescence was determined immediately after addition of the corresponding substrates.

LH_2-AMP obtained by chemical synthesis, and in good agreement with previous results [1].

LH_2-AMP from both sources was completely degraded after treatment with 4 μg/ml phosphodiesterase for 30 min.

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

The enzymatic formation of 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 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 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), 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 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.

Acknowledgements: This investigation was supported by grants from the Dirección General de Investigación Científica y Técnica (PM95/13). A.D. is a Visiting Professor in the Universidad Autónoma de Madrid, funded by the Dirección General de Investigación Científica y Técnica. We thank Isabel de Diego and Juan Ramón Serrano for able technical assistance.

References

[1] Rhodes, W.C. and McElroy, W.D. (1958) J. Biol. Chem. 233, 1528–1537.
[2] Denburg, J.L., Lee, R.T. and McElroy, W.D. (1969) Arch. Biochem Biophys. 134, 381–394.
[3] DeLuca, M. and McElroy, W.D. (1974) Biochemistry 13, 921–925.
[4] DeLuca, M. and McElroy, W.D. (1984) Biochem. Biophys. Res. Commun. 123, 764–770.
[5] Branchini, B.R., Hayward, M.M., Bamford, S, Brennan, P.M. and Lajiness, E.J. (1989) Photochem. Photobiol. 49, 689–695.
[6] Lembert, N. (1996) Biochem. J. 317, 273–277.
[7] Guranowski, A., Sillero, M.A.G. and Sillero, A. (1990) FEBS Lett. 271, 215–218.
[8] Sillero, M.A.G., Guranowski, A. and Sillero, A. (1991) Eur. J. Biochem. 202, 507–513.
[9] Ortiz, B., Fernández, V.M., Sillero, M.A.G. and Sillero, A. (1995) J. Photochem. Photobiol. B: Biol. 29, 33–36.
[10] Morton, R.A., Hopkins, T.A. and Seliger, H.H. (1969) Biochemistry 8, 1598–1607.
[11] Segel, I.H. (1975) Enzyme Kinetics, Wiley, New York.
[12] Lundin, A., Rickardsson, A. and Thore, A. (1976) Anal. Biochem. 75, 611–620.