pH opposite effects on synthesis of dinucleoside polyphosphates and on oxidation reactions catalyzed by firefly luciferase

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Received 11 February 2003; revised 9 April 2003; accepted 10 April 2003

First published online 18 April 2003

Edited by Judit Ovádi

Abstract Previous results have shown that an oxidizing product of firefly luciferin, dehydroluciferyl-adenylate, is the main intermediate in the process of synthesis of dinucleoside polyphosphates catalyzed by firefly luciferase (EC 1.13.12.7). However, we have found that the pH effects on the luciferase oxidizing processes and on the synthesis of dinucleoside polyphosphate are opposite: acidic assay media enhance the synthesis of dinucleoside polyphosphate and inhibit the oxidizing processes. The reason for this apparent contradiction lies on the activation effect of low pH on the adenylate transfer reaction from dehydroluciferyl-adenylate to the acceptor nucleotide.

Key words: Adenosine(5′)tetraphospho(5′)adenosine; Dehydroluciferin; Dinucleoside polyphosphate; Firefly luciferase; Luciferin

1. Introduction

The interest of the study of firefly luciferases is emphasized by the fact that the bioluminescent luciferase-catalyzed reaction is the basis for a wide variety of biochemical assays and that the luciferase gene is widely used as a reporter in studies of gene expression and regulation [1–3].

The bioluminescent reaction involves the formation, from firefly luciferin (LH2) and adenosine triphosphate (ATP), of an enzyme (E) bound adenylyl intermediate and its subsequent oxidation with release of adenylate (AMP), CO_2 and oxyluciferin, the presumed light emitter.

$$E + LH2 + ATP \rightarrow E{\cdot}LH2\text{-}AMP + \text{pyrophosphate} \quad (1)$$

$$E{\cdot}LH2\text{-}AMP + O_2 \rightarrow E + AMP + CO_2 + \text{oxyluciferin} + \text{photon} \quad (2)$$

Apart from oxyluciferin, dehydroluciferin (L) is also an oxidative product of LH2 [4–7]. Its formation from the luciferin moiety of the E·LH2-AMP complex was previously reported and its role on the characteristic decay of the firefly luciferase light production has been supported [4–6].

The cloning and sequencing of firefly luciferases have revealed that these enzymes are closely related to a large family of non-bioluminescent enzymes which also catalyze reactions of ATP with carboxylate substrates to form acyl-adenylated intermediates [1,8]. This group of proteins shares a common motif and has been termed the 'acyl-adenylate/thioester-forming' enzyme family [8].

Besides the production of light it has also been shown that luciferase of *Photinus pyralis* can catalyze the synthesis of adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) and other dinucleoside polyphosphates [9,10]; in addition, the same catalytic activity has been found in other members of the 'acyl-adenylate/thioester-forming' enzyme family [11,12]. In an attempt to clarify the luciferase mechanism of synthesis of dinucleoside polyphosphates it has been found that dehydroluciferyl-adenylate (L-AMP) is, at least in aerobic conditions, the main enzyme intermediate in this activity [6].

$$E{\cdot}L\text{-}AMP + \text{nucleoside polyphosphate } (p_nN) \rightarrow L + Ap_{(n+1)}N + E \quad (3)$$

To follow the synthesis of dinucleoside polyphosphates, relatively high concentrations of luciferase, luciferin or L and ATP are used. These conditions, although far away from the ones currently used in experiments of light emission, are presumably not very different from those found in nature. Besides, Ap_4A has been found in the lanterns of *P. pyralis*, indicating that their synthesis could be more than a simple artifact [13].

As stated above, firefly luciferase catalyzes two different oxidative processes that give rise to oxyluciferin and light or to L and synthesis of Ap_4A, but the conditions that enhance one of the pathways at the expense of the other remain unknown. In this report we will show that, although the oxidative processes that lead to the formation of both oxyluciferin and L are inhibited at low pH, this is a condition that increases the synthesis of Ap_4A. The explanation for this apparent contradiction lies on the activating effect of the low pH on the nucleoside-polyphosphate adenylate transfer step (Eq. 3).

2. Materials and methods

2.1. Materials

A stock solution of commercial luciferase (Sigma; L9506) was prepared by dissolving the lyophilized powder in deionized water (15 mg

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Abbreviations: Ap_4A, adenosine(5′)tetraphospho(5′)adenosine; L, dehydroluciferin; L-AMP, dehydroluciferyl-adenylate; LH2, luciferin; LH2-AMP, luciferyl-adenylate; PPase, inorganic pyrophosphatase; IP-HPLC, ion pair high performance liquid chromatography; RP-HPLC, reverse phase HPLC

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00382-X

lyophilisate/ml) and stored at −20°C. LH2, ATP, AMP, cytosine triphosphate (CTP) and inorganic pyrophosphatase (PPase) (I1891) were purchased from Sigma. L was chemically synthesized from 2-cyano-6-methoxybenzothiazole (Aldrich 943-03-3), converted into 2-cyano-6-methoxybenzothiazole-2-thiocarboxamide by treatment with hydrogen sulfide, pyridine and triethylamine [14]. The thioamide was condensed with methylbromopyruvate and the dimethyldehydroluciferin formed was treated with concentrated hydrobromic acid to give synthetic L; L was recrystallized from water. L-AMP was obtained from L and AMP as described previously [15] and purified by recrystallization from water.

2.2. Luciferase assays

All the enzyme reactions took place at ambient temperature and were performed in duplicate. After termination with the addition of solutions containing methanol (see below) the reaction mixtures were centrifuged for 1–2 min (13 400 rpm) and the supernatant injected into high performance liquid chromatography (HPLC) columns. The chromatographic system was constituted by a HP-1100 isocratic pump, a Rheodyne manual injection valve, silica-based octadecyl columns and a UNICAM Cristal 250 ultraviolet-visible (UV-VIS) diode array detector. For the separation of mono- and dinucleotides an ion pair (IP)-HPLC system was used: the eluent was a solution of 20% methanol, 20 mM tetrabutylammonium bromide and 40–75 mM sodium phosphate buffer (pH 7) and the flow rate was 0.5 ml/min. For the separation of LH2, L, L-AMP, and oxyluciferin a reverse phase (RP)-HPLC system was used: in this case the eluent was a mixture of methanol and sodium phosphate buffer (see below). The luminescence tests were performed with a homemade luminometer using a Hamamatsu HCL35 photomultiplier tube.

2.2.1. Enzyme synthesis of oxyluciferin and L at different pHs and light production. The assay media contained in a final volume of 50 μl: 100 μM ATP, 30 μM LH2, 2 mM $MgCl_2$, 100 mM buffer (MES pH 5.0 or pH 6.3 or HEPES pH 7.5) and luciferase (0.95 mg of protein/ml). The reactions were initiated by the addition of a mixture containing ATP, LH2, $MgCl_2$ and buffer to a solution of luciferase in front of the window of the photomultiplier tube; the light was measured continuously and 0.2 s intervals were integrated. At min 5 or min 30 the reactions were stopped by the addition of 100 μl of a mixture containing methanol (48%; v/v) and 10 mM ethylenediamine tetraacetic acid (EDTA). In the controls this mixture was added to luciferase before the other compounds. 50 μl aliquots of the stopped reaction mixtures were analyzed by an RP-HPLC system using, as eluent, a solution of 32% methanol (v/v) with 2 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min.

2.2.2. Synthesis of Ap_4A at different pHs using L or LH2 as cofactors. The standard reaction mixtures for the synthesis of Ap_4A contained in the final volume of 40 μl: 300 μM ATP, 30 μM LH2 or L, 2 mM $MgCl_2$, PPase (0.5 U/ml), 100 mM buffer (MES pH 5.0 and pH 6.3 or HEPES pH 7.5) and luciferase (0.36 mg of protein/ml). At 10, 20 and 30 min of incubation, 10 μl aliquots were withdrawn from the reaction mixtures and added to a solution of 66% methanol (40 μl). 20 μl were injected and analyzed by the IP-HPLC system described above.

2.2.3. Synthesis of Ap_4C from L-AMP and CTP at different pHs. The reaction mixtures for the synthesis of Ap_4C contained in the final volume of 100 μl: 130 μM L-AMP, 500 μM CTP, 2 mM $MgCl_2$, 100 mM buffer (MES pH 5.0 or pH 6.3 or HEPES pH 7.5) and luciferase (0.36 mg of protein/ml). At 30, 60 and 90 min of incubation, 22 μl aliquots were withdrawn from the reaction mixtures and added to a solution of 66% methanol (88 μl). After centrifugation the supernatant was separated in two fractions of similar volume; one of the fractions was analyzed by the IP-HPLC system described above and the other by RP-HPLC. In this case, 20 μl aliquots were injected, the flow rate was 0.7 ml/min, and the eluent was a solution of 28% methanol (v/v) and 2 mM sodium phosphate buffer (pH 7). The controls (without luciferase) were incubated for 90 min.

2.2.4. Effect of CTP concentration at different pH values on adenylate transfer from L-AMP (Ap_4C synthesis). The reaction mixtures contained in the final volume of 30 μl: 130 μM L-AMP, luciferase (0.71 mg of protein/ml), 100 mM buffer (MES pH 5.0 or pH 6.3 or HEPES pH 7.5), CTP at different concentrations ranging from 100 μM to 8 mM and $MgCl_2$ (1 mM in excess over CTP concentration). In order to define the initial rate, 3 μl aliquots were withdrawn from reaction mixtures and added to 66% methanol (27 μl) at different times of incubation. After centrifugation the supernatant was analyzed by the IP-HPLC system described above.

3. Results and discussion

Experimental evidence supporting the idea that the main intermediate in the luciferase-catalyzed synthesis of dinucleoside polyphosphates in aerobic conditions is L-AMP has already been presented [6]. This implies that the enzyme activity of synthesis of dinucleoside polyphosphates relies on the previous oxidation of LH2-AMP to L-AMP. Results from other authors have shown that, in contrast to the synthesis of Ap_4A that is favored by acidic pH (optimum pH of 5.7) [10], light production is best at pH above 7 [16]. This previous knowledge led us to test if the activating effect of acidic pH on the synthesis of dinucleoside polyphosphates was a consequence of a stimulating effect of acidification on the pathway that oxidizes the LH2 moiety of LH2-AMP into L.

Using HEPES (pH 7.5) or MES (pH 6.3 or 5.0) we began studying the effect of pH on the synthesis of L, oxyluciferin and light production. As expected, light production and synthesis of oxyluciferin dropped with decreasing pH (Figs. 1 and 2). However, in opposition with the current knowledge on the role of L-AMP in Ap_4A synthesis and on the pH effect on this activity, we found that the formation of L was favored at basic pH (the corresponding chromatographic peak was hardly seen at pH 5.0 as observed in Fig. 2). Although we could not anticipate this result, it was not a complete surprise. As was the case for the formation of oxyluciferin [1] the oxidative conversion of LH2-AMP to L-AMP may occur at the stage of the deprotonation of C4 of the luciferyl moiety; acidification may protonate the luciferase residue(s) involved in this deprotonation thus inhibiting both oxidation reactions.

With that knowledge we proceeded studying the effect of the same pH values on the synthesis of Ap_4A using LH2 or L as added cofactors either in the presence or absence of PPase. Confirming previous results [6,9] the synthesis of Ap_4A was residual when LH2 was the added cofactor, PPase was absent and pH was 7.5 (Fig. 3). The dramatic activating effect of PPase when LH2 was the added cofactor and pH was 7.5

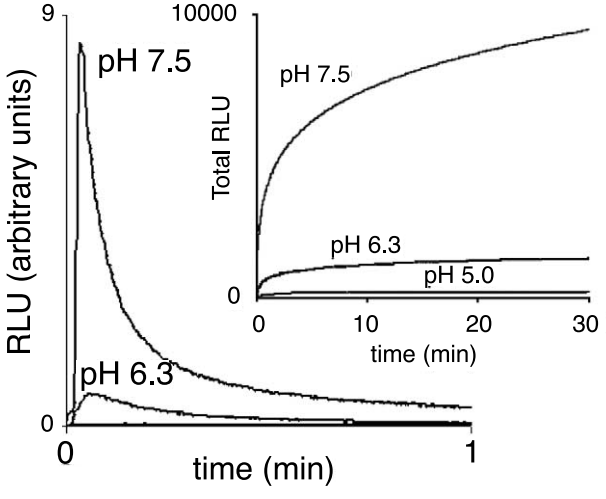

Fig. 1. Light production in reaction mixtures containing buffer (MES pH 5.0 or 6.3 or HEPES pH 7.5), ATP, LH2, $MgCl_2$ and luciferase at the indicated pH values. See Section 2 for details.

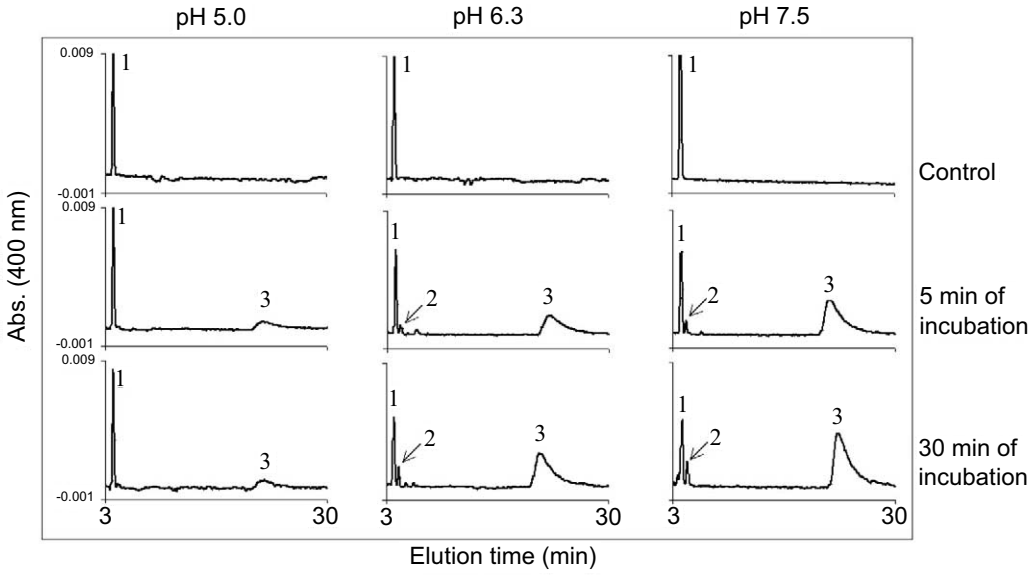

Fig. 2. Chromatograms obtained by RP-HPLC analysis of bioluminescent reaction mixtures performed at the indicated pH values and stopped at the indicated times of incubation. 1, 2 and 3 on top of the chromatographic peaks correspond to LH2, L and oxyluciferin, respectively. See Section 2 for details.

was also confirmed; further it was observed that a similar result could be obtained at pH 6.3 but not at pH 5.0 (Fig. 3). As expected, added for similar concentrations, L was better than LH2 at all the studied pH values (Fig. 3). The most intriguing result was the activating effect of pH 5.0 relative to the other two pHs: in all the chosen conditions this effect was evident and, except when LH2 was the added cofactor and PPase was present, the activity of synthesis of Ap_4A increased more than two times when the pH was decreased from 6.3 to 5.0 (Fig. 3).

The finding that the synthesis of Ap_4A and the synthesis of L changed in opposite directions when the pH was decreased from 6.3 to 5.0 was a stimulating challenge: it was strange that the formation of the reported main intermediate was residual at the pH in which the synthesis of Ap_4A occurred best. Trying to find an explanation we have hypothesized that the lower pH could modify the enzyme accelerating the process of transfer of the adenylate moiety of the intermediate to the acceptor nucleotide (Eq. 3). To test this hypothesis we chemically synthesized L-AMP and used it to study the adenylate transfer reaction from L-AMP to the nucleotide acceptor CTP (Fig. 4). Confirming our previous assumptions we found that the pH profile of the synthesis of Ap_4C from L-AMP and CTP was very similar to that corresponding to the synthesis of Ap_4A in conditions where the steps of formation of the intermediate were not bypassed (Figs. 3 and 5). Apparently, the low pH stimulating effect on the final step of synthesis of Ap_4A more than compensates its inhibitory effect on the oxidative and/or on the LH2 adenylation steps.

The results presented above demonstrate that the limiting step on the synthesis of dinucleoside polyphosphates is the adenylate transfer reaction from L-AMP to the nucleotide acceptor and that acidic media activate this step.

The activating effect of acidic pH could result from a decrease on the K_m of CTP or an increase of k_{cat}. At pH 5.0, the K_m of CTP was estimated as 0.54 mM and the k_{cat} as 2.9 min^{-1}. In the range of CTP concentrations used (0.1–8 mM) the reaction rates at pH 7.5 and 6.3, always lower than those at pH 5.0, were proportional to the substrate concentration. Although we were not able to calculate the K_m and k_{cat} at the more basic pHs, our results showed that pH 5.0 causes an increase on the affinity of CTP for the enzyme, therefore accelerating the process of adenylate transfer.

We advance here that this could be related with a change in Lase configuration, possibly to a more open active center. This hypothesis is in line with other experimental evidences

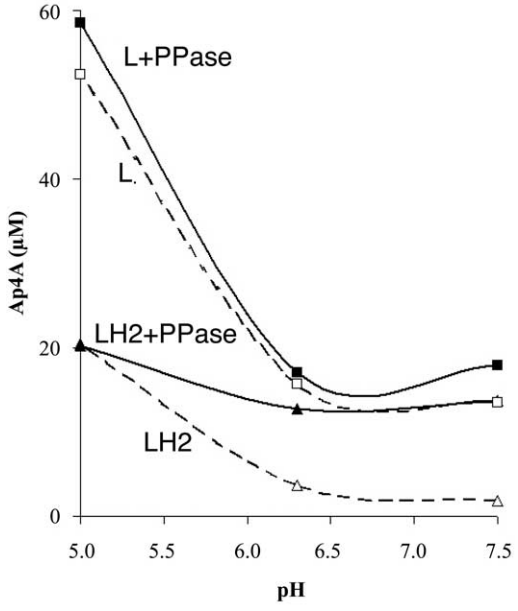

Fig. 3. Luciferase-catalyzed synthesis of Ap_4A at the indicated pH values, performed in the presence (L+PPase; LH2+PPase) or in the absence (L; LH2) of PPase using LH2 or L as added cofactors and stopped at 20 min of incubation. Each point represents an average of two experiments. See Section 2 for details.

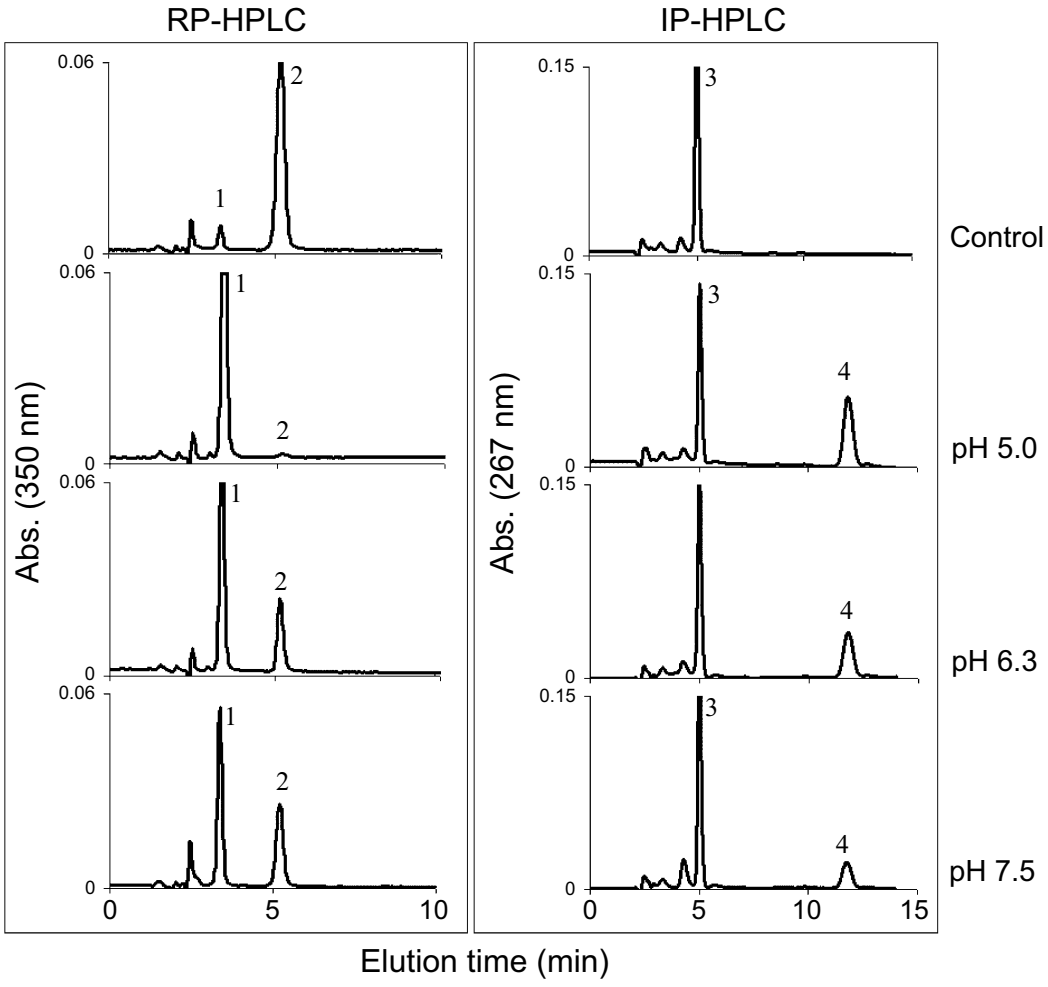

Fig. 4. Luciferase-catalyzed synthesis of Ap_4C at the indicated pH values using L-AMP as adenylate donor and CTP as acceptor. The presented chromatograms were obtained by RP-HPLC and IP-HPLC analysis of aliquots taken from reaction mixtures stopped at 90 min of incubation. Peaks 1, 2, 3 and 4 correspond to L, L-AMP, CTP and Ap_4C, respectively. L was present in the control because it contaminated our L-AMP preparation. See Section 2 for details.

that emphasize the importance of enzyme structure on the red shift of the light emitted at acidic pHs [17,18].

The activating effect of acidification on the synthesis of dinucleoside phosphates reported here could be useful in biotechnology: actually, firefly luciferase has already been used to obtain a variety of dinucleoside tetraphosphates useful in the study of HIV-1 reverse transcriptase [19]. The quantitative modification of the activities of luciferase at acidic pHs may

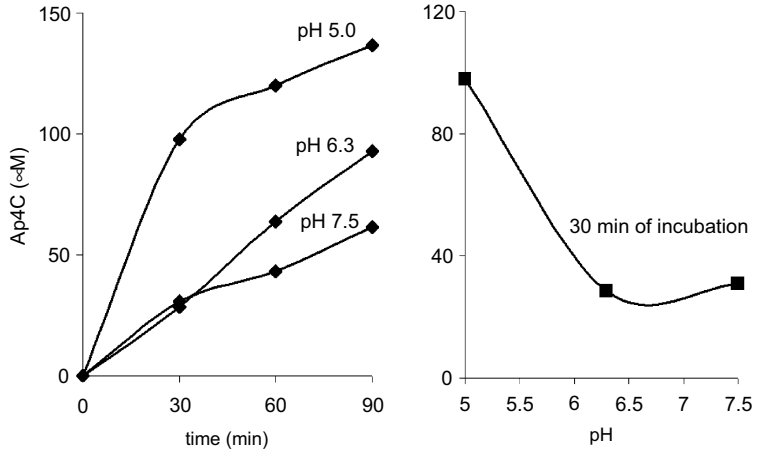

Fig. 5. Time course and pH profile of the luciferase-catalyzed synthesis of Ap_4C at the indicated pH values using L-AMP as adenylate donor and CTP as acceptor. Each point represents an average of two experiments. See Section 2 for details.

be a first step in the discovery of unsuspected qualitative modifications (including specificity change) that may reveal luciferase as a tool for the synthesis of other useful compounds.

Acknowledgements: Financial support from FCT (FSE-FEDER) (project POCTI/QUI/37768/2001) is acknowledged.

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