FEBS Letters 438 (1998) 190–194 FEBS 21058

Dehydroluciferyl-AMP is the main intermediate in the luciferin dependent synthesis of Ap_4A catalyzed by firefly luciferase

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Received 9 September 1998

Abstract It was previously assumed that E·LH_2-AMP was the intermediate complex in the synthesis of Ap_4A catalyzed by firefly luciferase (EC 1.13.12.7), when luciferin (LH_2) was used as cofactor. However, here we show that LH_2 is partly transformed, shortly after the onset of the luciferase reaction, to dehydroluciferin (L) with formation of an E·L-AMP complex which is the main intermediate for the synthesis of Ap_4A. Formation of three more derivatives of LH_2 were also observed, related to the production of light by the enzyme. CoA, a known stimulator of light production, inhibits the synthesis of Ap_4A by reacting with the E·L-AMP complex and yielding L-CoA.

Key words: Ap_4A; Luciferase; Luciferin; Dehydroluciferin; Coenzyme A

1. Introduction

Firefly luciferase (EC 1.13.12.7) catalyzes the production of light using luciferin (LH_2) and ATP as cofactors.

$$E + LH_2 + ATP \rightarrow E{\cdot}LH_2{-}AMP + PP_i \quad (1)$$

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

The production of light starts with an initial flash that usually decays in few seconds to low basal levels, even in the presence of excess luciferin and ATP [1]. Depending on the relative concentrations of enzyme, luciferin, ATP and the nature of the buffers and metals present in the reaction mixture, almost any kind of kinetics can be observed [2]. The interpretation of these phenomena is still not clear. The E·LH_2-AMP complex may follow at least two pathways: toward production of light (Eq. 2) and toward E·L-AMP formation [3,4]. As L-AMP binds very strongly to luciferase [5] the formation of the E·L-AMP complex contributes to the inhibition of light production by sequestering active enzyme molecules [4]. In another respect, the activating effect of CoA in the luminescent reaction has been interpreted as a consequence of its reaction with the L moiety of the E·L-AMP complex yielding L-CoA and liberating enzyme, allowing it to recycle with further light production [3–5].

Firefly luciferase also catalyzes the synthesis of diadenosine tetraphosphate (Ap_4A) according to the previously proposed general reaction [6]

$$E{\cdot}LH_2{-}AMP + ATP \rightarrow E + Ap_4A + LH_2 \quad (3)$$

Using other nucleoside triphosphates (NTP) or P_3 as acceptors of the AMP moiety luciferase catalyzes the synthesis of Ap_4N and p_4A, respectively [7]. As Eq. 1 is freely reversible, the synthesis of Ap_4A is greatly accelerated by pyrophosphatase [6]. Contrary to light emission (see above) the rate of synthesis of Ap_4A is constant along time (min or hours) depending on the availability of the substrate ATP [8]. Dehydroluciferin and 6′-ethyl-luciferin (both non-oxidizable compounds by luciferase) are also cofactors in the synthesis of dinucleoside polyphosphates [8].

The object of this work was to explore further the role of luciferin and dehydroluciferin and the effect of CoA on the synthesis of Ap_4A catalyzed by luciferase. The main outcomes are that (i) in the presence of oxygen and luciferin, the main intermediate in the synthesis of dinucleoside polyphosphates is E·L-AMP and not, as previously assumed, E·LH_2-AMP; (ii) CoA, activator of the production of light, is a strong inhibitor of the synthesis of dinucleoside polyphosphates.

2. Materials and methods

2.1. Materials

Luciferase from firefly (*Photinus pyralis*) was purchased from Sigma (catalogue no. L-5226). A stock solution (4.5 mg of protein/ml) of luciferase was dissolved in 0.5 M HEPES/KOH (pH 7.5). D-Luciferin (LH_2), ATP, sodium tripolyphosphate (P_3), and CoA were obtained from Sigma. Inorganic pyrophosphatase (EC 3.6.1.1; 200 U/ml) was from Boehringer Mannheim. [α-^{32}P]ATP was from DuPont NEN. Dehydroluciferin (L) was prepared from D-luciferin as previously described [8,9]. The thin layer chromatography (TLC) UV_{254} silica gel fluorescent plates were from Merck.

2.2. Luciferase assay

The reaction mixtures contained, unless otherwise indicated, 100 mM HEPES/KOH (pH 7.5), 2 mM $MgCl_2$, 0.1 mM ATP, PPase (0.5%; v/v), 20–35 μM dehydroluciferin or luciferin, and luciferase 0.45–0.9 mg of protein/ml. Incubations were carried out at 30°C. Product formation was analyzed by HPLC or by TLC.

2.2.1. HPLC. Reaction mixtures (0.05 ml) were stopped by the addition of 0.1 ml of 10 mM EDTA in 66% methanol and centrifuged for 1–2 min at 13 000 rpm. Aliquots of the supernatant (0.05 ml) were injected into a Hypersil ODS column (4.6×100 mm) and eluted at a constant flow rate (0.5 ml/min) with 2 mM phosphate buffer (pH 7) in 32% methanol. Chromatograms were registered simultaneously at the following wavelengths: 260, 327, 348 and 385 nm. The calculations made to transform the areas of the chromatographic peaks into concentrations were based on the relative molar absorbance of luciferin

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Abbreviations: Ap_4A, adenosine(5′)tetraphospho(5′)adenosine; L, dehydroluciferin; L-AMP, dehydroluciferyl-adenylate; Lase, luciferase; L-CoA, dehydroluciferyl-coenzyme A; LH_2, luciferin; LH_2-AMP, luciferyl-adenylate; P_3, tripolyphosphate; p_4A, adenosine 5′-tetraphosphate; PPase, pyrophosphatase

PII: S0014-5793(98)01301-5

and dehydroluciferin at, respectively, 327 and 348 nm [10], and on the relative molar absorbance of dehydroluciferin and L-AMP at 353 nm [5]. To calculate the concentration of L-CoA it was assumed that at 348 nm of wavelength the molar absorbance of L-CoA was identical to that of L-AMP.

2.2.2. TLC. The reaction mixtures (0.02 ml), as above, were supplemented with 0.1 μCi of [α-^{32}P]ATP. At the indicated times aliquots (3 μl) were spotted on TLC plates. The plates were first developed (13 cm) in dioxane/50 mM acetic acid (4:1) and, after drying, developed again (10 cm) in dioxane/ammonium hydroxide/water (6:1:5). The fluorescent spots were observed using light at a wavelength of 366 nm, and the radioactivity measured in an InstantImager (from Packard Instruments). Standards of non-radioactive AMP, Ap_4A, ADP, ATP and p_4A were eluted simultaneously.

3. Results

3.1. Synthesis and characterization of dehydroluciferyl-AMP

Reaction mixtures containing [α-^{32}P]ATP, luciferase and dehydroluciferin or luciferin were incubated in the absence or in the presence of pyrophosphatase, and analyzed by TLC (Fig. 1). The synthesis of Ap_4A was higher in the presence of L than in the presence of LH_2 and, in both cases, stimulation by pyrophosphatase was evident, particularly after 30 min of incubation (Fig. 1). On the contrary, synthesis of AMP was clearly observed only in the presence of LH_2; its rate of synthesis (irrespective of the presence or absence of pyrophosphatase) was very high at the onset of the reaction and then decreased sharply. In addition to the expected spots corresponding to ATP and ADP (contaminating the [α-^{32}P]ATP preparations) a new radioactive compound (spot 'a'), fluorescent when irradiated with light of 366 nm wavelength, and migrating just in front of markers of L and LH_2, was also observed. The radioactivity in spot 'a', more intense with L than with LH_2, did not change markedly during the time of incubation. Spot 'a' could correspond to an intermediate of the luciferase catalyzed reactions rather than to a product. The nature of spot 'a' was approached testing the reactivity of the presumed intermediate with P_3 and CoA (Fig. 2). When dehydroluciferin was used (lanes 1–4) the compound corresponding to spot 'a' disappeared in the presence of P_3 or CoA giving place to p_4A and AMP, respectively (Fig. 2). The results obtained with luciferin (lanes 5–8) were very similar (apart from the synthesis of AMP in the absence of added CoA, already observed in the experiment described in Fig. 1). Since formation of LH_2-CoA has never been reported, in our view spots 'a' (Figs. 1 and 2) should correspond mainly to L-AMP.

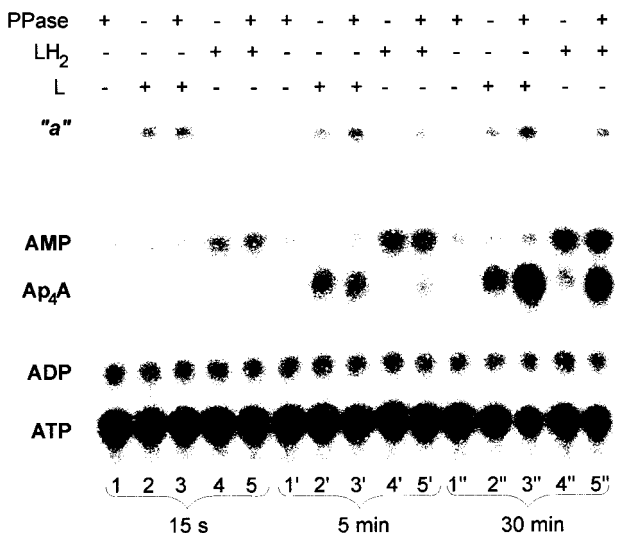

Fig. 1. Synthesis of L-AMP, AMP and Ap_4A catalyzed by firefly luciferase. Effect of dehydroluciferin, luciferin and pyrophosphatase. Reaction mixtures containing [α-^{32}P]ATP, luciferase (0.9 mg of protein/ml), 30 μM L or LH_2, and pyrophosphatase (PPase), as indicated, were incubated at 30°C. At 15 s, 5 min and 30 min aliquots were taken and spotted on TLC. The plates were developed and the radioactivity measured as described in Section 2. Elution position of non-radioactive standards of AMP, ADP, ATP and Ap_4A are shown. Lanes 1, 1′ and 1″ correspond to a control without either L and LH_2; 'a' presumptive L-AMP.

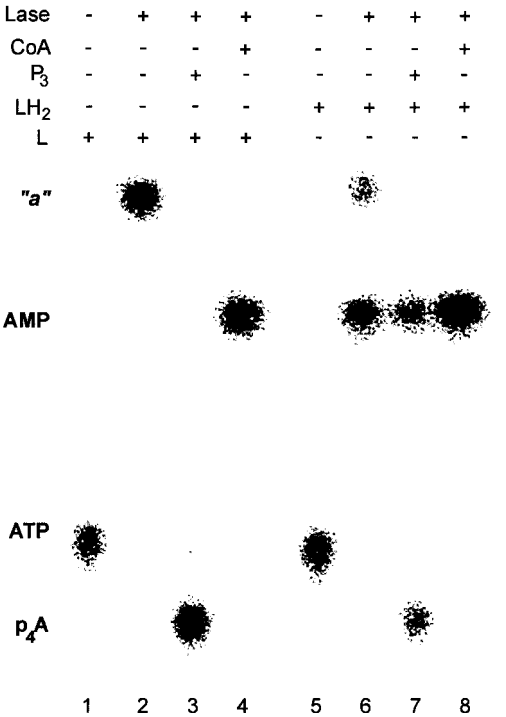

Fig. 2. Luciferase catalyzed reaction. Radioactive products formed in the presence of P_3 or CoA. Reaction mixtures containing [α-^{32}P]ATP (3000 Ci/mmol; about 0.01 μM), PPase, 20 μM L or LH_2, 5 mM $MgCl_2$, and 4 mM P_3 or 0.1 mM CoA were incubated in the presence or in the absence of luciferase (0.68 mg of protein/ml) for 10 min. The TLC plate was developed and the radioactivity counted as described in Section 2. Elution position of non-radioactive AMP, ATP and p_4A are indicated; 'a' presumptive L-AMP. Lanes 1 and 5: Controls without enzyme.

3.2. Analysis by HPLC of the synthesis of L and L-AMP catalyzed by luciferase: effect of pyrophosphatase

To better identify the reaction products or intermediates, reaction mixtures containing luciferase, ATP and dehydroluciferin or luciferin were incubated for 30 min in the absence or presence of pyrophosphatase and analyzed by HPLC as described in Section 2. When dehydroluciferin was used as cofactor, it gave rise to a new peak (result not shown) identified as corresponding to L-AMP by its absorption spectrum [11] (Fig. 3). When luciferin was used as cofactor, it gave rise to (among other compounds, see Fig. 4) dehydroluciferin and L-AMP. These two products were easily identified by their elution position and absorption spectra coincident either with chemically synthesized dehydroluciferin or with the L-AMP synthesized by luciferase from dehydroluciferin and ATP (Fig. 3). No peak corresponding to LH_2-AMP could be observed (results not shown). The final concentrations (μM) of

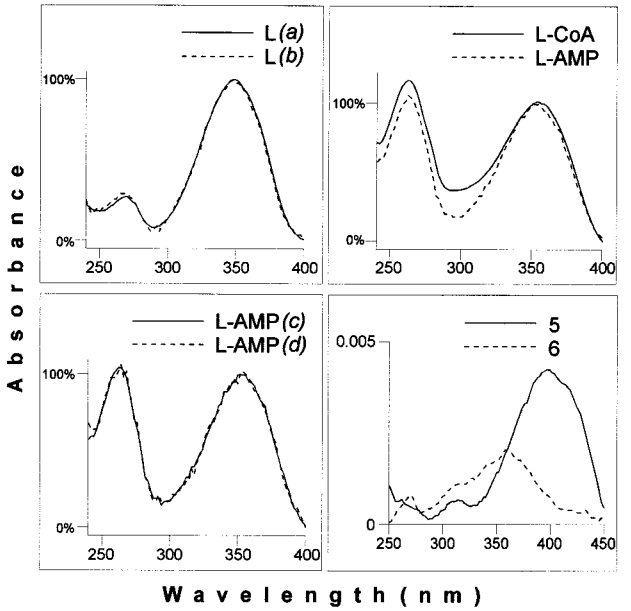

Fig. 3. UV absorption spectra of L, L-AMP, L-CoA and other products formed during the firefly catalyzed reaction. *a* and *b* correspond to the spectra of L chemically or enzymatically synthesized, respectively; *c* and *d* are the spectra of L-AMP enzymatically synthesized from L or from LH_2, respectively. These spectra and that of L-CoA were normalized to a relative absorbance of 100% at 348 nm (L) or 354 nm (L-AMP and L-CoA). Spectra of compounds 5 and 6 were obtained from analysis of reaction mixtures containing luciferase and luciferin as the ones described in Fig. 4.

L-AMP, after 30 min incubation, in the assays with L or (LH_2) were 4.4 (0.8) and 7.8 (3.4), in the absence and presence of pyrophosphatase, respectively. In both cases the presence of pyrophosphatase increased the level of L-AMP in the reaction mixture. The above values were in good agreement with the calculated concentrations of [α-^{32}P]AMP in spots 'a' in Fig. 1.

3.3. Time course of the products formed during the luciferase catalyzed reaction

The analysis by HPLC of the luciferase assay performed in the presence of LH_2 showed that L+L-AMP account for only around 16% of the consumed LH_2, the rest being transformed to other products as a consequence of the luminescent reaction. To follow the time course of the UV absorbing products formed, reaction mixtures containing ATP, luciferin and luciferase were incubated in the absence and presence of pyrophosphatase, and analyzed by HPLC for more prolonged elution times (Fig. 4). In addition to the peaks corresponding to LH_2, L and L-AMP, other UV absorbing products of the reaction (4, 5 and 6) were now detected in these new experimental conditions. Pyrophosphatase greatly affected the relative concentration of L and L-AMP enhancing the formation of the last. Peaks 4 and 6 reached a maximum in the first minute of the reaction declining during the incubation time, whereas peak 5 appears later in the course of the reaction. This behavior was independent of the presence or absence of pyrophosphatase. The spectra of compounds 5 and 6 are shown in Fig. 3. The absorbance of peak 4 was too low to obtain a good spectrum, although its maximum seemed to be around 400 nm (not shown). All spectra where taken in elution buffer (2 mM phosphate buffer (pH 7) in 32% methanol).

3.4. Effect of CoA on the concentration of dehydroluciferyl-AMP and on the synthesis of Ap_4A

It was known that while CoA activated the luminescent reaction [3,4], it inhibited the synthesis of Ap_4A (unpublished results from this laboratory and this work). Since, in our view, this finding seemed to be related to the level of L-AMP present in the reaction, we tried to analyze, simultaneously, the effect of CoA on the synthesis of Ap_4A and on the level of L-AMP. The experiment was performed in the presence of [α-^{32}P]ATP, luciferase, pyrophosphatase, fixed concentrations (35 μM) of luciferin or dehydroluciferin and variable concentrations of CoA (0–0.1 mM) (Fig. 5). While, in the presence of L, a concentration of 0.025 mM CoA inhibited only slightly the synthesis of Ap_4A, the same amount of CoA almost abolished the synthesis of Ap_4A in the presence of LH_2. The CoA dependent inhibitory profile of the synthesis of Ap_4A and the level of L-AMP could be superimposed both with LH_2 or L as cofactors (Fig. 5). This was further tested analyzing by HPLC the relative amounts of L, L-AMP and L-CoA, in the presence of increasing concentrations of CoA in the assays where the added cofactor was L (Fig. 5, inset). L-CoA eluted just before L and its UV spectrum (Fig. 3) was similar to that of L-AMP although not identical. With increasing concentrations of CoA, L-CoA increased and L decreased, and the concentration of L-AMP almost did not change as long as the added CoA was in defect compared to the L present. Both L and L-AMP disappeared, and were converted to L-CoA, when CoA (100 μM) was in large excess relative to L (35 μM).

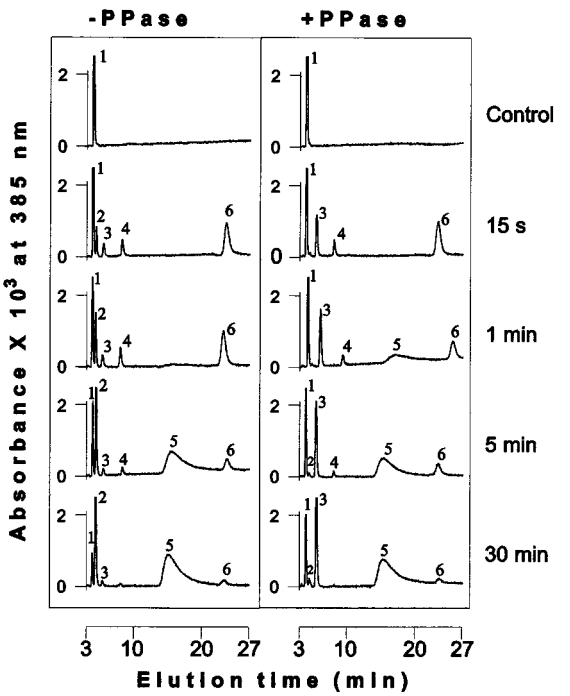

Fig. 4. Time course of the products formed during luciferase catalyzed reaction in the presence of luciferin and ATP. Reaction mixtures (0.25 ml) containing ATP, luciferase (0.9 mg of protein/ml) and 30 μM LH_2 were incubated in the absence or presence of PPase. At 15 s, 1, 5 and 30 min aliquots were taken and analyzed by HPLC as described. The figures 1, 2 and 3 on top of the chromatographic peaks correspond to LH_2, L and L-AMP, respectively; 4, 5 and 6 correspond to products related to light production.

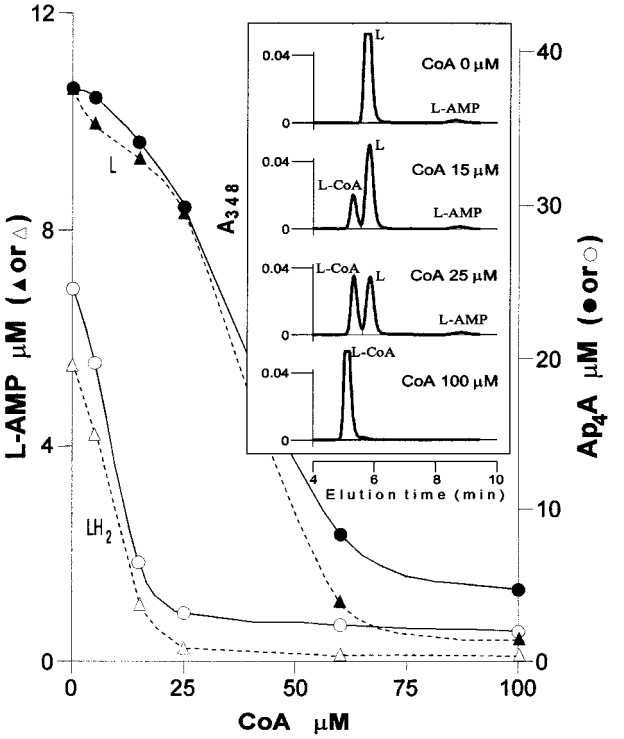

Fig. 5. Effect of CoA on the synthesis of Ap_4A and L-AMP catalyzed by luciferase in the presence of dehydroluciferin or luciferin. Reaction mixtures containing [α-^{32}P]ATP, luciferase (0.9 mg of protein/ml), PPase, 35 μM L (full symbols) or LH_2 (empty symbols) and CoA as indicated were incubated for 1 h and analyzed by TLC as described. The concentrations of L-AMP (triangles) and Ap_4A (circles) represent the average of two experiments. Inset: Effect of increasing concentrations of CoA on the relative levels of L, L-AMP and L-CoA in the luciferase catalyzed reaction. Reaction mixtures containing 0.5 mM ATP, luciferase (0.45 mg of protein/ml), PPase, 35 μM of L and the indicated concentrations of CoA were incubated for 30 min. The reactions were analyzed by HPLC as described.

4. Discussion

During the last years, firefly luciferase, ATP, other nucleoside triphosphates and polyphosphates of different chain length were used for the synthesis of a variety of (di)nucleoside polyphosphates [6,7,12]. In general luciferin was used as cofactor and the reaction analyzed by HPLC, after prolonged times of incubation, usually hours. In those experiments, little attention was paid either to the kinetics of light production (that could be observed by the naked eye) or to the fate of luciferin in the course of the reaction, and the synthesis of dinucleoside polyphosphates was thought to take place through formation of the E·LH_2-AMP complex (Eq. 3). However, here we show that, even using LH_2 as cofactor, the main intermediate donor of AMP is the E·L-AMP complex, rather than E·LH_2-AMP.

With the help of a sensitive method (using radioactive [α-^{32}P]ATP), the synthesis of Ap_4A could be correlated with the appearance of a compound ('a' in Figs. 1 and 2) that was later identified as L-AMP (see below). In the presence of L, this compound appeared at the very onset of the reaction, its level increased only slightly during incubation time and the presence of pyrophosphatase increased both its steady state level and the amount of Ap_4A synthesized. In the presence of LH_2 two main differences were appreciated: (i) rapid synthesis of AMP, due to the oxidation of the E·LH_2-AMP complex, concomitant with the production of light and the production of oxyluciferin, and (ii) less synthesis of Ap_4A and a lower level of the presumptive L-AMP, as compared to the results obtained with L. Again, in the case of LH_2, the steady state level of the presumptive L-AMP was related to the amount of Ap_4A synthesized.

The fate of LH_2 was approached analyzing the reaction mixtures by HPLC (Fig. 4). Peaks corresponding to L and L-AMP were formed as well as three other new peaks, termed 4, 5 and 6 (Fig. 4), one of which could correspond to oxyluciferin [13–15]. Apparently, pyrophosphatase had little effect on the rate of formation of compounds 4, 5 and 6, but it greatly affected the relative concentrations of L and L-AMP enhancing the formation of the latter (Fig. 4). A compound with spectral characteristics compatible with LH_2-AMP that eluted in the same elution position as L, was observed in very low amounts (less than 0.4 μM) in assays performed in the presence of LH_2 and CoA, when almost no L was present (results not shown). The low concentrations of LH_2-AMP attained in the luciferase reactions are in line with previous results from this laboratory [16]. Although CoA is a strong inhibitor of the synthesis of Ap_4A in the presence of LH_2 as cofactor, the synthesis of Ap_4A could not be completely abolished, even in the presence of high concentrations of CoA (see Fig. 5), due, probably, to the presence of low concentrations of the E·LH_2-AMP intermediate which in the presence of ATP could give rise to Ap_4A. The fact that 6′-ethyl-luciferin, a non-oxidizable analogue of luciferin, is also a cofactor in the synthesis of Ap_4A [8] demonstrates that at least one complex other than E·L-AMP could react with ATP in the synthesis of Ap_4A.

The different effect of CoA on light production (stimulator) and on the synthesis of Ap_4A (inhibitor), reported here for the first time, can now be easily explained. As CoA reacts with the E·L-AMP complex generating L-CoA and AMP (Figs. 2 and 5) and free enzyme, luciferase can enter in a second cycle of light production, as far as LH_2 and ATP are available. The inhibitory effect of CoA on the synthesis of Ap_4A is more effective with LH_2 than with L as a consequence of the lower levels of L and L-AMP attained when the cofactor added to the reaction mixtures was LH_2. With dehydroluciferin the inhibition was observed clearly only when the concentration of added CoA was in excess over the cofactor while with luciferin the inhibitory effect was observed even when the added CoA was in defect. In both cases, the rate of synthesis of Ap_4A was correlated with the steady state concentration of L-AMP (Fig. 5). The concentration of L-AMP remains almost constant, despite addition of CoA, if sufficient dehydroluciferin remains in the assay media to react with ATP and maintain the steady levels of the intermediate in the synthesis of Ap_4A, L-AMP (Fig. 5).

Acknowledgements: This investigation was supported by a grant (PM 95/13) from the Dirección General de Investigación Científica y Técnica. R.F. was partially supported by a Fellowship from Junta Nacional de Investigação Científica e Tecnológica.

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