Identification of Luciferyl Adenylate and Luciferyl Coenzyme A Synthesized by Firefly Luciferase

Hugo Fraga,[a] Joaquim C. G. Esteves da Silva,*[a] and Rui Fontes[b]

The firefly luciferase reaction intermediate luciferyl adenylate was detected by RP-HPLC analysis when the luciferase reaction was performed under a nitrogen atmosphere. Although this compound is always specified as an intermediate in the light-production reaction, this is the first report of its identification by HPLC in a luciferase assay medium. Under a low-oxygen atmosphere, luciferase can catalyze the synthesis of luciferyl coenzyme A from luciferin, ATP, and coenzyme A, but in air dehydroluciferyl coenzyme A was produced. The luciferase-catalyzed synthesis of these coenzyme A derivatives may be a consequence of the postulated recent evolutionary origin of firefly luciferases from an ancestral acyl-coenzyme A synthetase.

Introduction

Luciferyl adenylate (LH_2-AMP) is reported to be formed during the *Photinus pyralis* luciferase-catalyzed reaction involving D-luciferin (LH_2) {(*S*)-2-[6′-hydroxy-2′-benzothiazolyl]-2-thiazoline-4-carboxylic acid} and ATP as subtracts, and Mg^{2+} as an essential cofactor (Scheme 1). Although LH_2-AMP has been referred to for a long time as the active intermediate of firefly luciferase (Lase) reactions[1–5] and its chemical synthesis has been described,[1, 2] the direct identification of the enzymatic LH_2-AMP has not in our opinion been clearly demonstrated. In the firefly luciferase bioluminescent reaction, LH_2-AMP is oxidized by O_2 with the subsequent release of AMP, CO_2, and oxyluciferin {2-[6′-hydroxy-2′-benzothiazolyl]-4-hydroxythiazole}, the light emitter (Scheme 1).

The light emission starts with an initial flash that is either maintained for a particular period of time or decays in a few seconds to a low basal level. Depending on the relative concentration of Lase, ATP, and a variety of other conditions, different types of kinetics may be obtained.[3, 4, 6, 7]

Supplementation of standard assay media with coenzyme A (CoA) increases initial light emission and retards the usual light decay.[6, 7] Light decay is thought to be caused by slow release of reaction products from the enzyme active site, and the effect of CoA has been interpreted as a consequence of its action as a promoter of product removal.[6–8] The mechanism of the stimulatory effect of CoA is controversial, but the lack of any effect from dethioCoA, a CoA analogue lacking the sulfur atom, emphasizes the importance of the thiol group.[7] Firefly Lase shares some primary structural motifs with other enzymes (grouped in the "acyl adenylate/thioester-forming" enzyme family) that catalyze the formation of carboxylate-CoA thioesters,[9–12] and it can catalyze the formation of dehydroluciferyl CoA (L-CoA) in reaction mixtures containing ATP, CoA, and dehydroluciferin (L) {2-[6′-hydroxy-2′-benzothiazolyl]-thiazole-4-carboxylic acid}.[3, 5, 6, 8] Actually, the similarities between firefly Lase and fatty acyl-CoA synthetases' catalytic mechanisms were emphasized as early as 1967, by McElroy et al.[13]

Dehydroluciferyl AMP (L-AMP) is a potent inhibitor of light emission and it, like oxyluciferin, is a compound formed from LH_2-AMP in the course of the bioluminescence reaction (Scheme 1).[1, 5, 6, 8] These data and the lack of a carboxyl group in oxyluciferin (excluding it from reaction with CoA) were the basis for the proposal that the stimulatory effect of CoA on luminescence depends on its reactivity with L-AMP, forming L-CoA and liberating the enzyme from bound L-AMP (Scheme 1).[5, 8]

Although the obvious structural analogies between LH_2-AMP and L-AMP point to the possibility that luciferyl CoA (LH_2-CoA) could also be formed in the course of the reaction, this hypothesis, already advanced by McElroy et al.[13] and by Wood,[3] has, as far as we know, never been tested.

On analyzing Lase assay media by reversed-phase HPLC (RP-HPLC), we were able to demonstrate unequivocally the formation of the enzyme intermediate LH_2-AMP from ATP and LH_2 and to study the effect of various factors on its steady-state concentration. Moreover, the luciferase-catalyzed formation of LH_2-CoA from ATP, LH_2, and CoA was also observed and its enzymatic synthesis studied (Scheme 1).

While this manuscript was being written, a paper describing luciferase activity in the synthesis of long chain acyl-CoA appeared.[14] This result and ours provide new evidence in support of the theory of the evolutionary relationship between Lase and an ancestral acyl-CoA synthetase.[3]

[a] *Dr. H. Fraga, Prof. J. C. G. Esteves da Silva*
LAQUIPAI, Departamento de Química
Faculdade de Ciências, Universidade do Porto
R. Campo Alegre 687, 4169-007 Porto (Portugal)
Fax: (+351) 22-6082959
E-mail: jcsilva@fc.up.pt

[b] *Prof. R. Fontes*
Serviço de Bioquímica (U38-FCT)
Faculdade de Medicina, Universidade do Porto
Alameda Hernâni Monteiro, 4200 – 319 Porto (Portugal)

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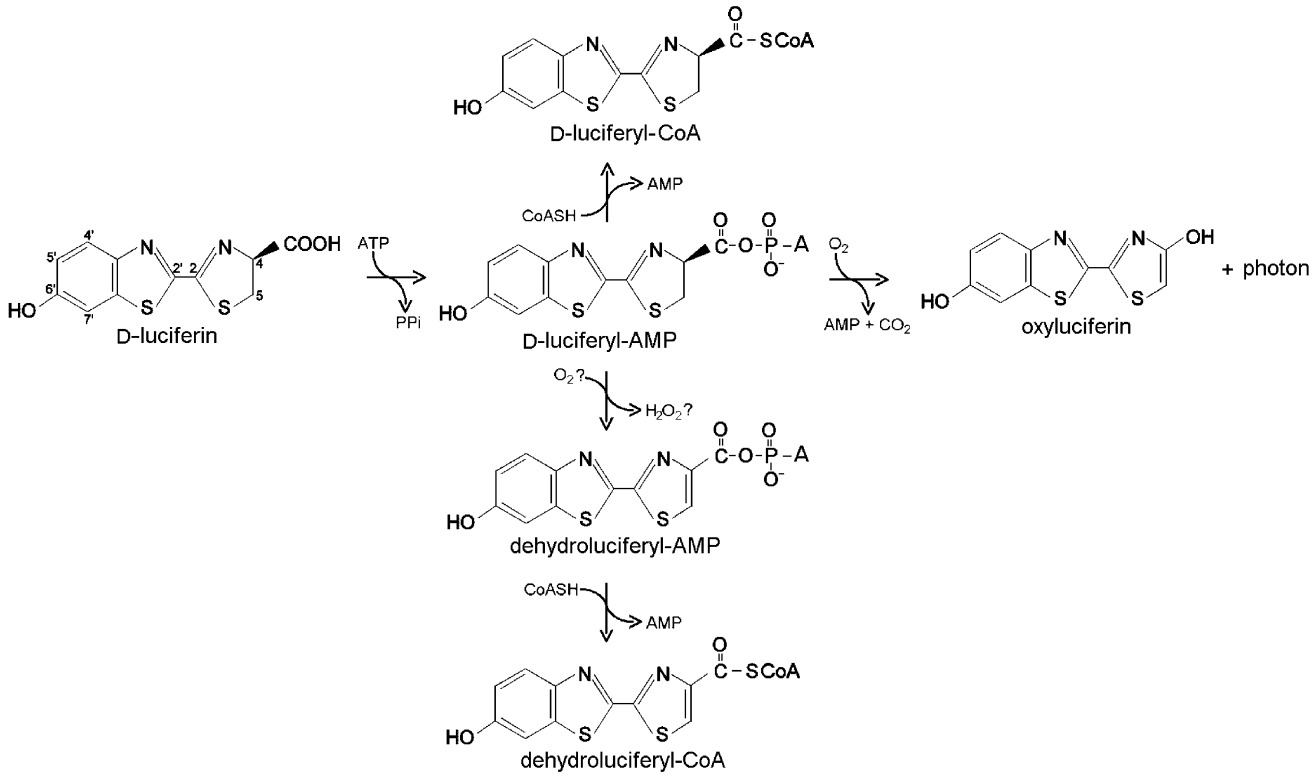

Scheme 1. *Diagram showing the reactions catalyzed by firefly luciferase. A, adenosine.*

Results and Discussion

Firefly luciferase-catalyzed synthesis of LH_2-AMP

On the basis of the proposed reaction mechanism of the firefly luciferase reaction, it was deduced that the concentration of the enzyme intermediate LH_2-AMP could be maximized if: 1) PPi (pyrophosphate; the other product in its formation from ATP and LH_2) was removed by PPase (pyrophosphatase), 2) the oxidation of LH_2-AMP was prevented by the use of anaerobic conditions, and/or 3) its stabilization was favored by high concentrations of Lase.

The effect of these three factors was confirmed: analysis of reaction mixtures stopped by the addition of methanol after two minutes' reaction time under N_2, with PPase present and high Lase concentration, showed the formation of two chromatographic peaks (P1 and P2). These peaks eluted immediately after LH_2 and their spectra were coincident with two maxima at 336 and 262 nm (Figure 1). Chromatographic peaks with the same spectra and elution times were also observed in reaction mixtures in which the chemical synthesis of LH_2-AMP was performed (Figure 1). The chemical epimerization of synthetic luciferyl adenylates had already been described[2] and we suspected that the natural D isomer was isomerizing at pH 7.5 after the enzyme reaction had been stopped. Analysis of the stopped reaction mixtures immediately after the removal of the precipitated proteins by centrifugation allowed the identification of P1 (the isomer eluting first) as the enantiomer formed by the enzyme: D-LH_2-AMP (Figure 1). The epimerization rate could also be slowed down by decreasing the pH: when the pH was 6.3, even after 30 min of delay, the quantity of P2 (L-LH_2-AMP) formed was minimal. We observed similar results for the chemically synthesized LH_2-AMP.

The purification and lyophilization of the chemically synthesized compounds corresponding to P1 and P2 allowed us to test their capacities to produce light when mixed with Lase and oxygen. To minimize epimerization and hydrolysis, the pH of the eluent used in the purification procedure was 5.6 and the light-production reactions were performed at pH 6.3. Confirming P1 as the natural D isomer (D-LH_2-AMP), bright light was produced on treatment with luciferase, whilst the light was almost undetectable when it was substituted by purified P2 (Figure 1 C).

Factors affecting the enzymatic synthesis of LH_2-AMP

After confirming the identity of P1, we proceeded to study the effect of oxygen, PPase, and Lase concentration on its accumulation. When air was bubbled through the reaction mixture, the luciferyl adenylates were no longer detected. It is pertinent to relate that the accumulation of "an active intermediate" had already been shown to take place under anaerobic conditions in elegant experiments done by Hastings et al. more than fifty years ago.[15] In those experiments it was shown that the intensity of the flash of light obtained upon admission of oxygen into a previously hypoxic reaction mixture containing LH_2, ATP, $MgCl_2$, and Lase increased inversely with the percentage of oxygen during the preincubation.

In our experiments we observed that air bubbling, apart from preventing the detection of luciferyl adenylates, also increased a

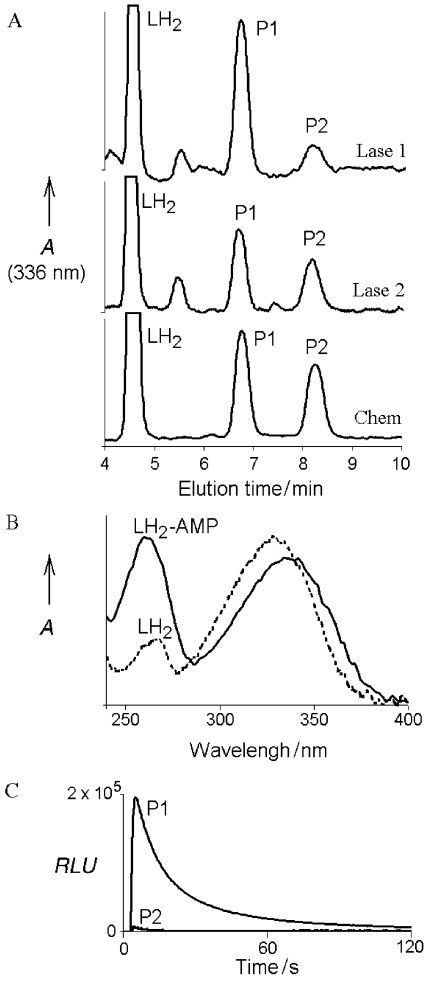

Figure 1. *Identification of luciferase-synthesized LH_2-AMP. A) Chromatograms of enzyme preparations analyzed without delay after the reaction had been stopped (Lase 1) and 10 minutes later (Lase 2), and of the chemically synthesized LH_2-AMP (Chem). The chromatographic peaks P1 and P2 correspond to D-LH_2-AMP and L-LH_2-AMP enantiomers, respectively. B) Absorbance spectra of LH_2 and LH_2-AMP. C) Light production (RLU, relative light units) with purified P1 and P2.*

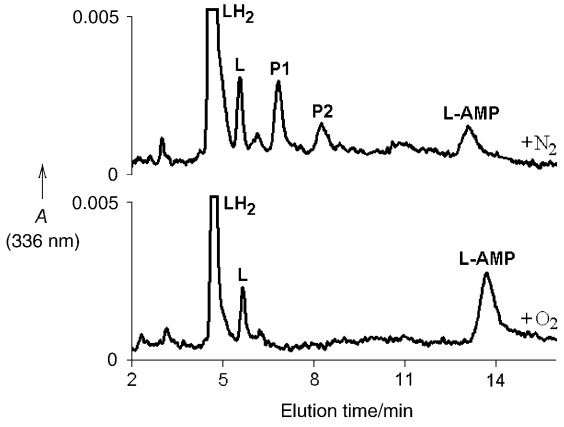

Figure 2. *Chromatograms of enzyme preparations prepared under nitrogen ($+N_2$) or under air ($+O_2$). The chromatographic peaks P1 and P2 correspond to D-LH_2-AMP and L-LH_2-AMP enantiomers, respectively.*

chromatographic peak that was identified as corresponding to L-AMP (Figure 2). Its identification was obtained through injection, under the same elution conditions, of the chemically synthesized compound. Actually, L-AMP, an oxidation product of LH_2-AMP, was formed even under N_2 (Figure 2); we presume that the formation of L-AMP was due to the presence of traces of O_2 in the reaction mixture. Under the elution conditions used to separate LH_2-AMP from LH_2, the retention time of oxyluciferin, the light-emitter oxidation product, is too long,[16] and, as would be expected, it was not detected.

The presence of inorganic pyrophosphatase (PPase) eliminates PPi, the other product in the step of formation of LH_2-AMP (Scheme 1). The addition of PPase, although not essential for the detection of LH_2-AMP, markedly increased its concentration in the reaction media (not shown).

By varying the amount of enzyme added to the assay mixtures we found that, as expected, the concentration of the accumulated D-LH_2-AMP was almost proportional to the amount of Lase: at pH 6.3 its concentrations were estimated to be 5.8, 11, and 14 μM when the concentrations of protein were 0.6, 1.2 and 1.8 mg mL^{-1}, respectively.

By withdrawing aliquots from fully aerobic bioluminescent reaction mixtures (containing Lase, LH_2, $MgCl_2$, and ATP) when the flash of light had already gone out, and by accepting the light that was produced when those aliquots were mixed with fresh luciferase as a measure of the LH_2-AMP present in the aliquots, Dukhovich et al.[17] have studied the pH dependence of LH_2-AMP formation. From those experiments they concluded that the maximal activity for luciferase-catalyzed LH_2-AMP accumulation was reached at pH 7.8. As already described, we were unable to find LH_2-AMP by our detection method under fully aerobic conditions. Under N_2 the concentration of LH_2-AMP was higher at pH 6.3 and 7.0 than at pH 7.5 and 8.3 (Figure 3 A).

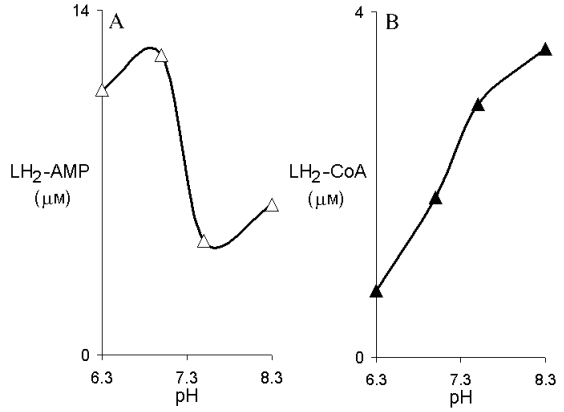

Figure 3. *Effect of pH on A) the steady-state concentration of LH_2-AMP and on B) the enzyme synthesis of LH_2-CoA.*

As we could not exclude the presence of traces of O_2, these concentrations were the result of the rate constants and the concentrations of reactants involved on its formation and oxidation, and changing the pH of the assay may affect both of the processes. The influence of the assay time on the concentration of LH_2-AMP was also studied: as expected for a

true intermediate, its steady-state concentration was maintained almost constant throughout the first 15 min of assay (Figure 4).

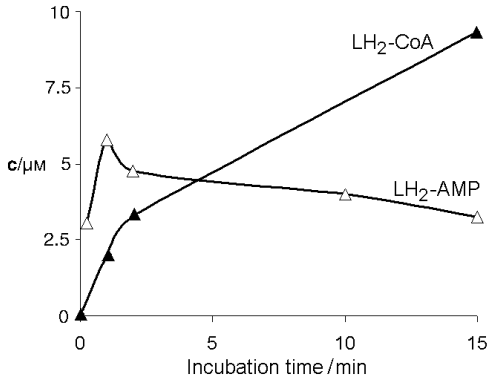

Figure 4. *LH_2-AMP and LH_2-CoA concentrations at different incubation times.*

Firefly luciferase-catalyzed synthesis of LH_2-CoA

Having established the conditions for the detection of LH_2-AMP, we tested its reactivity with CoA in the presence of ATP, LH_2, Lase, and PPase under a N_2 atmosphere. A new compound, eluting before LH_2, was formed (Figure 5 A), and we suspected that it corresponded to LH_2-CoA. As expected, its spectrum was similar to that of LH_2-AMP, with two maxima at 336 and 262 nm (Figure 5 B); the similarity of the spectra of their oxidized partners, L-CoA and L-AMP, had already been reported[5] and was confirmed in this work. In contrast with the case of LH_2-AMP, the concentration of LH_2-CoA increased with incubation time (Figure 4); LH_2-CoA is therefore not an intermediate, but a product. In contrast with the observation of two LH_2-AMP enantiomers, only one chromatographic peak was observed for LH_2-CoA, which probably corresponds to the D-LH_2-CoA [(R)-LH_2-CoA] isomer. Nevertheless, the detection of only one chromatographic peak does not demonstrate that chemical epimerization {D-LH_2-CoA $\rightleftharpoons$ L-LH_2-CoA} does not occur, because the chromatographic system may have no resolving capacity for the enantiomers of LH_2-CoA.

When the assays were performed in air the peak corresponding to LH_2-CoA decreased sharply (or did not appear at all), and instead, another peak, eluting between LH_2 and L-AMP, could be detected (Figure 5 A). From its spectrum we suspected that it could correspond to L-CoA, the oxidized partner of LH_2-CoA, but its elution time was different from the one reported in the literature.[5] Its definitive identification as L-CoA was achieved when we compared its elution time with that of the compound formed in assay mixtures containing L-AMP, CoA and Lase under the current analysis conditions. As expected, when dethioCoA was used instead of CoA neither LH_2-CoA nor L-CoA was formed (Figure 5 A). The existence of thioester bonds between CoA and the luciferyl and dehydroluciferyl moieties of LH_2- and L-CoA derivatives was indicated by the non-reactivity of dethioCoA with LH_2-AMP and L-AMP.

We tested the effect of Mg^{2+} on the Lase-catalyzed transfer reaction of the L moiety of chemically synthesized L-AMP to CoA

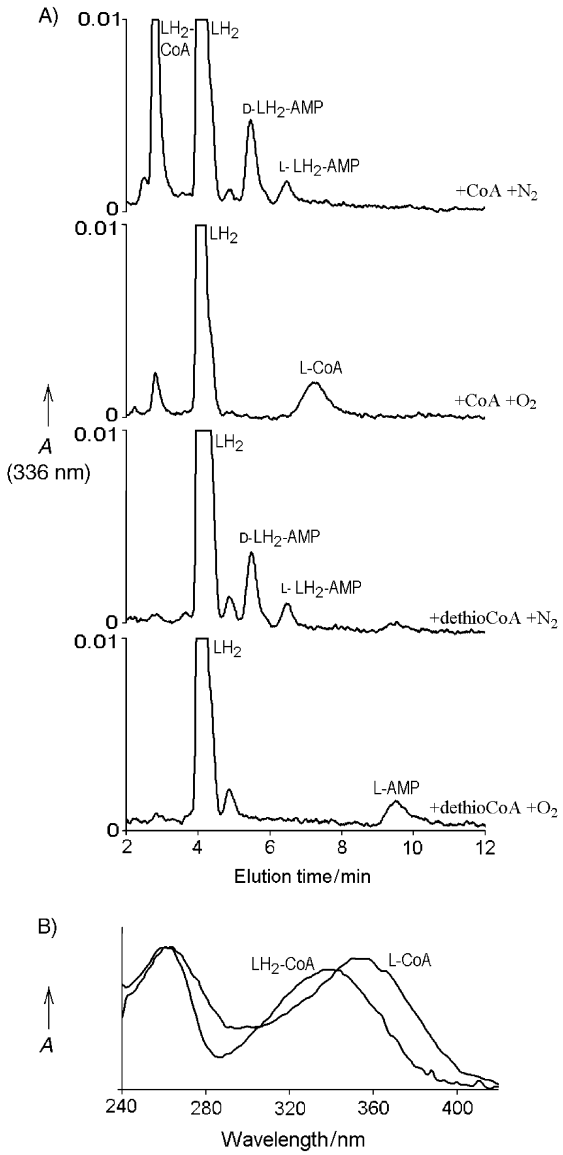

Figure 5. *Enzyme synthesis of LH_2-CoA from LH_2, ATP, and CoA. A) Chromatograms of enzyme preparations prepared under nitrogen (+ N_2) or under room atmosphere (+ O_2) as indicated. B) Absorbance spectra of LH_2-CoA and L-CoA.*

and found that, in this reaction, it was not an essential cofactor. This result is in line with the observation that Mg^{2+} is also unnecessary in the similar acetyl-transfer reaction from acetyl-AMP to CoA, catalyzed by acetyl-CoA synthetase.[18] From the resemblance of the compounds LH_2-CoA and L-CoA and their respective formation reactions (Scheme 1), we postulate that the metallic ion, although essential for the activation steps in which ATP participates,[1, 18] do not participate in the LH_2-AMP-CoA transfer step. It has been known for more than forty years that the same applies for the oxidation steps in which oxyluciferin is formed.[1]

A peak with the same elution time as LH_2-CoA could sometimes be observed when the Lase assays containing CoA were performed in the presence of oxygen (see Figure 5 A). Unfortunately its absorbance was too low to provide a spectrum that could be unequivocally identified as corresponding to LH_2-

CoA. Although the enzyme synthesis of LH_2-CoA seemed to depend on anaerobic conditions under our experimental and analysis conditions, the possibility of its formation in the presence of oxygen cannot definitively be excluded. In this case, as postulated by Wood,[3] it may have a role in the activating effect of CoA in light production. We are aware that the role of CoA on the Lase bioluminescent reaction remains somewhat obscure and that its clarification needs more work.

The pH profile for the formation of LH_2-CoA (Figure 3B) contrasted with that observed in the case of the steady-state concentration of LH_2-AMP (Figure 3A) but was similar to the light quantum yield pH profile of Lase,[19] and increased from pH 6.3 to pH 8.3.

As already emphasized by Wood,[3] firefly Lase may have evolved from an ancestral acyl-CoA synthetase. The similarities between the primary sequences of the active site of Lase and acyl-CoA synthetases[9–12] and the fact that they share the capacity to catalyze the synthesis of dinucleoside polyphosphates[20–23] and various acyl-CoA derivatives[5, 6, 14] are remarkable. The fact that luciferase still retains an acyl-CoA synthetase activity for its natural substrate, LH_2, may be attributed to the postulated recent evolutionary origin of beetle bioluminescence.

Experimental Section

Materials: Stock solutions of commercial Lase (Sigma; L9506) and commercial PPase (Sigma; I1891) were prepared by dissolving the lyophilized powder in deionized water (15 mg lyophilisate per mL and 100 units per mL, respectively) and stored at $-20\,^{\circ}C$. LH_2, ATP, AMP, and dethioCoA were also purchased from Sigma, and CoA (27593) from Fluka. D- and L-LH_2-AMP enantiomers were chemically synthesized from LH_2 and AMP as described previously,[24] and purified by RP-HPLC (with 20% methanol and 0.35 mM sodium phosphate pH 5.6 as eluent). L was chemically synthesized from 2-cyano-6-methoxybenzothiazole (Aldrich 943-03-3), and was converted into 2-cyano-6-methoxybenzothiazole-2-thiocarboxamide by treatment with hydrogen sulfide, pyridine, and triethylamine.[25] The thioamide was condensed with methyl bromopyruvate, and the dimethyldehydroluciferin formed was treated with concentrated hydrobromic acid to give synthetic L, which was then recrystallized from water. L-AMP was obtained from L and AMP as described previously[24] and purified by recrystallization from water.

Firefly luciferase-catalyzed synthesis of LH_2-AMP: The standard reaction mixtures (15 μL) for the synthesis of LH_2-AMP used ATP (0.5 mM), LH_2 (30 μM), $MgCl_2$ (2 mM), PPase (1.5 μL stock solution), HEPES (100 mM, pH 7.0), and Lase (1 – 1.7 mg protein per mL). All the solutions were prepared and kept under N_2 atmosphere. The reactions were initiated by the addition of Lase and were performed at ambient temperature (20 °C). After 2 min of incubation the enzyme reaction was stopped by the addition of 30 μL of 66% methanol (*v*/*v*) and centrifuged (13400 rpm for 1 min), and the supernatant was analyzed without delay by RP-HPLC, with injection of 20 μL aliquots (Supercosil LC-18, 150 × 4.6 mm, 3 μm; purchased from Supelco). Elution was performed at a constant flow rate of 1 mL min^{-1} with a solution of a phosphate buffer (2 mM, pH 7.0) in 25 – 29% methanol (*v*/*v*). The detector was a UV/Vis diode array (ATI UNICAN Crystal 250).

When the effect of the presence of oxygen was tested (Figure 2) the concentration of LH_2 was 60 μM, the assay pH 7.5, and the incubation time 10 min. When the effect of pH was tested (Figure 3A) the buffer was MES for pH 6.3 and HEPES for the other pH values (7.0, 7.5, or 8.3) and the incubation time was 1 min.

Bioluminescence from the synthetic D- and L-LH_2-AMP enantiomers: The bioluminescence tests were performed with a homemade luminometer using a Hamamatsu HCL35 photomultiplier tube. The reaction mixtures contained, in a final volume of 40 μL, 10 μM chemically synthesized and purified D-LH_2-AMP or L-LH_2-AMP, MES (100 mM, pH 6.3), and Lase (0.95 mg protein per mL). Light was measured for 5 min with integration of 0.2 s intervals.

Firefly luciferase-catalyzed synthesis of LH_2-CoA: The standard assay and analysis conditions for the synthesis of LH_2-CoA were identical to those described above for the case of the enzymatic synthesis of LH_2-AMP, except that the reaction mixture was supplemented with CoA (0.1 mM), the assay pH was 7.5, and the incubation time was 5 min.

When the effect of the presence of oxygen was tested (Figure 5) the concentration of LH_2 was 60 μM and the incubation time 10 min; when dethioCoA was used its concentration was 0.1 mM. When the effect of pH was tested (Figure 3B) the buffer was MES for pH 6.3 and HEPES for the other pH values (7.0, 7.5, or 8.3), the concentration of Lase was 0.34 mg protein per mL, and the incubation time was 5 min.

Firefly luciferase-catalyzed synthesis of L-CoA from L-AMP and CoA: The assay mixtures contained CoA (100 μM), L-AMP (30 μM), Hepes (100 mM, pH 7.5), and Lase (0.035 mg of protein per mL). The incubation time was 30 s and the analysis conditions were as referred to above.

Calculation of concentrations: The calculations made to transform the areas of the chromatographic peaks into concentrations were based on the relative molar absorbance of the pertinent substances.[1, 24] In the case of LH_2-CoA it was assumed that its molar absorbance was the same as that of LH_2-AMP.

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