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Chemical synthesis and firefly luciferase produced dehydroluciferyl-coenzyme A

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Abstract—Dehydroluciferyl-coenzyme A (L-CoA) was chemically synthesized and characterized by MS, UV–vis spectrometry and RP-HPLC. The identity of the chemically synthesized compound with the one that was produced by firefly luciferase was confirmed. Moreover, the reversibility of the enzymatic conversion of dehydroluciferin $\rightleftharpoons$ dehydroluciferyl-adenylate $\rightleftharpoons$ L-CoA was also confirmed. The chemical synthesis of L-CoA, described here, may help the clarification of the activator effect of CoA on luciferase bioluminescent assays, in which the enzyme catalyzed formation of L-CoA and the consequent destruction of L-AMP is one of the possible explanations for that effect.

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Firefly luciferase is an enzyme that catalyzes the oxidation of luciferin (LH_2){(*S*)-2-[6′-hydroxy-2′-benzothiazolyl]-2-thiazoline-4-carboxylic acid} (Fig. 1) in the presence of ATP and magnesium ion giving rise to light.[1] The bioluminescent reaction involves the formation, from LH_2 and ATP, of an enzyme bound adenylyl intermediate (LH_2-AMP)[2,3] (reaction 1) and its subsequent oxidation with release of AMP, pyrophosphate (PPi), CO_2 and oxyluciferin {2-[6′-hydroxy-2′-benzothiazolyl]-4-hydroxythiazole} (Fig. 1) (reaction 2), the presumed light emitter.[4] Apart from oxyluciferin, dehydroluciferin (L) {2-[6′-hydroxy-2′-benzothiazolyl]-thiazole-4-carboxylic acid} is also an oxidative product of LH_2. Its formation from the luciferin moiety of the E·LH_2-AMP was previously reported and its role on the characteristic decay of the firefly light production has been supported.[5]

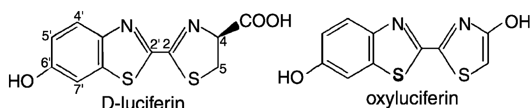
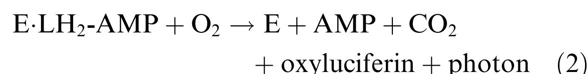


Figure 1. Chemical structures of luciferin and oxyluciferin.

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

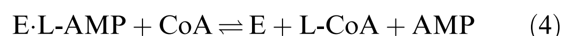


$$E{\cdot}LH_2\text{-}AMP + O_2 \rightarrow E + AMP + CO_2 + \text{oxyluciferin} + \text{photon} \quad (2)$$

The supplementation of reaction media with coenzyme A (CoA) results in an enhancement in the light production and in a delay in the characteristic inhibition profile. In the course of the bioluminescent reaction L-AMP and L are also produced, and the activating effect of CoA on firefly light emission 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.[2,5–7]

As pointed by McElroy et al.[8] in 1968, the mechanism of synthesis of L-CoA by luciferase (reactions 3 and 4) may be similar to that of fatty acid activation by acyl-CoA synthetases.

$$E + L + ATP \rightleftharpoons E{\cdot}L\text{-}AMP + PPi \quad (3)$$



$$E{\cdot}L\text{-}AMP + CoA \rightleftharpoons E + L\text{-}CoA + AMP \quad (4)$$

The suspected structural relationship between firefly luciferase and acyl-CoA synthetases was confirmed by molecular biology studies in the last decade;[1] these and other enzymes are now grouped under the denomination of acyl-adenylate/thioester-forming enzyme family.

Keywords: Dehydroluciferyl-coenzyme A; Firefly luciferase; Coenzyme A; Bioluminescence.

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Despite the relevance of the enzymatic process of synthesis of L-CoA, the unequivocal assignment of L-CoA was never done mainly because the chemical synthesis of this compound has never been achieved.

With the objective of unequivocally identify the luciferase synthesized L-CoA the chemical synthesis of this compound was undergone by modifying a procedure employed for the synthesis of acyl-CoA derivatives,[9] (Fig. 2). The synthesis started with the dissolution of chemically synthesized L[10] in tetrahydrofuran (THF) and left to react overnight with carbonyldiimidazole at room temperature.[11] The activated dehydroluciferin was left to react with CoA in an alkaline medium for one day.[11] Purification was performed by RP-HPLC.[12] The purified compound was characterized by MALDI mass spectroscopy (Fig. 3, panel A) and RP-HPLC-DAD (UV–vis, Fig. 3, panel B). The mass spectra confirmed the expected mass for the compound formed from a thioester bound between L and CoA (calculated $M_w = 1027.83$ g/mol).

The identification of the luciferase synthesized L-CoA was obtained by RP-HPLC-DAD, using methodologies developed for the separation of compounds involved in the firefly luciferase catalyzed reactions.[3,4,7] Although luciferase is also able to catalyze the synthesis of L-CoA when, starting from LH_2 and ATP, the intermediate LH_2-AMP is oxidized to L-AMP, which then transfers the L moiety to CoA,[3] in this work chemically synthesized L-AMP was used as L donor (reaction 4). That is, the enzymatic synthesis was performed by incubating L-AMP and CoA in the presence of luciferase.[13] The observed chromatographic retention times and UV–vis spectrum for both chemically and enzyme synthesized L-CoA coincided (Fig. 4).

Airth et al.[6] were able to verify the reversibility of reactions 3 and 4 by observing the disappearance of L in reaction mixtures containing ATP, L, CoA and luciferase and its reformation when the assay media was supplemented with AMP and pyrophosphate (PPi). The entire process was monitored by fluorescence, taking advantage of the fact that both L-CoA and L-AMP are nonfluorescent in contrast with L that exhibits strong fluorescence (excitation 353 nm; emission 540 nm). It should be noted that some confusion exists in luciferase related literature because, by that time, L and L-CoA were wrongly named as oxyluciferin (the light emitter) and oxyluciferyl-CoA, respectively.[2,5,6]

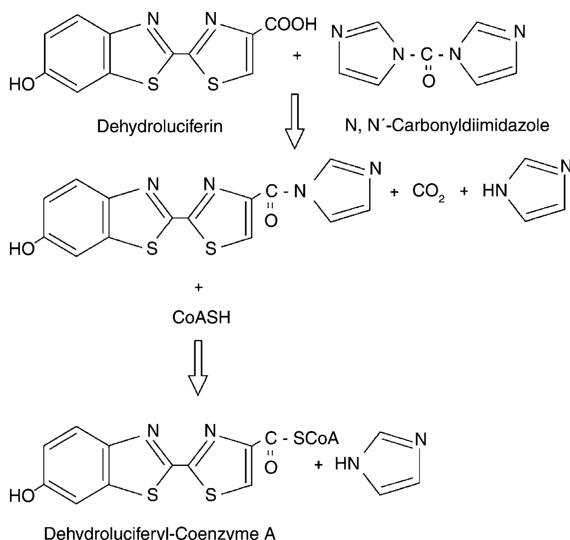

Figure 2. Scheme of chemical synthesis of L-CoA.

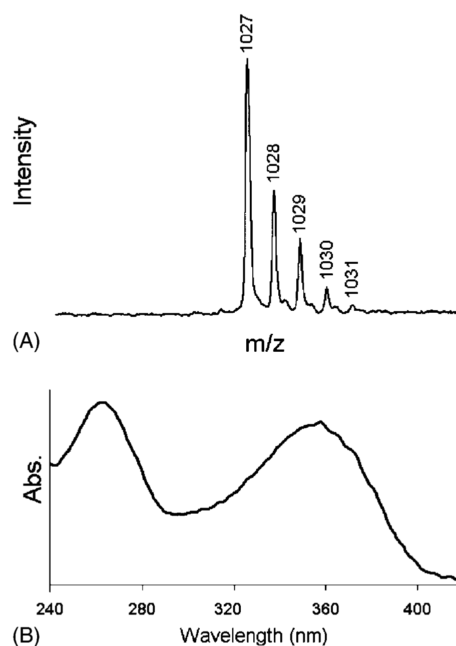

Figure 3. MALDI mass (A) and UV–vis (B) spectra of the chemically synthesized L-CoA.

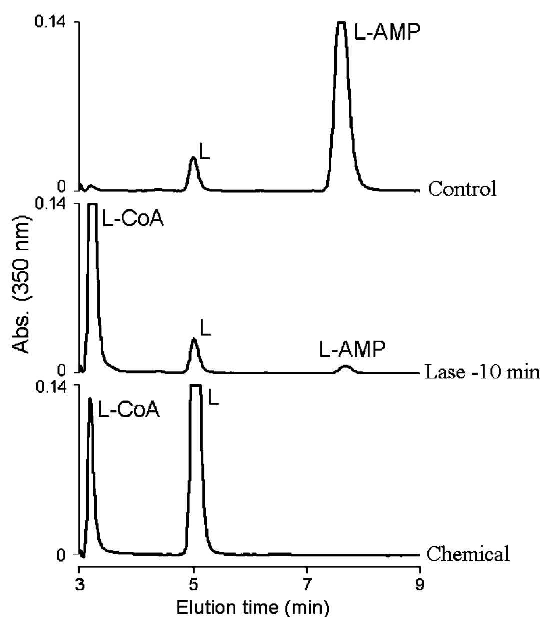

Figure 4. Enzyme and chemically synthesized L-CoA. Control corresponds to an assay similar to Lase-10 min where Lase was omitted.

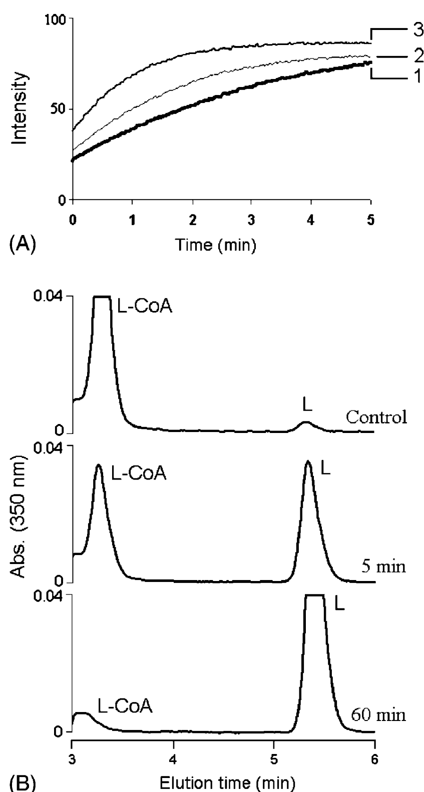

Figure 5. Luciferase catalyzed synthesis of L from L-CoA, AMP and PPi. Panel A—fluorescence profiles: curves 1, 2 and 3 correspond to different concentrations of luciferase; 0.011, 0.023 and 0.033 mg protein/mL, respectively. Panel B—chromatograms of the reaction mixture stopped at 5 and 60 min of incubation; control corresponds to an assay where luciferase was omitted stopped at 60 min.

We have checked the same reversibility using the chemically synthesized and purified L-CoA mixed with AMP, PPi and luciferase;[14] the reaction was monitored by fluorescence and also by RP-HPLC (Fig. 5). The chromatographic results showed that, in those conditions, L-CoA was converted into L (Fig. 5, panel B). When the reaction was monitored by fluorescence we confirmed the expected increase of fluorescence along the incubation time (Fig. 5, panel A) and the effect of luciferase concentration on the speed of the reaction.

The formation of L from L-CoA could not be attributed to hydrolysis and was catalyzed by luciferase: the reaction depended on the simultaneous presence of AMP, PPi and luciferase (reactions 4 and 3).

Using the same chromatographic methodologies[15] the pH dependence of the luciferase catalyzed synthesis of L-CoA either from ATP, L and CoA or from L-AMP and CoA was also studied. The obtained pH profiles (Fig. 6) showed that, in both cases, the enzyme activity had a maximum at about 7.5.

This is the first report of the chemical synthesis of L-CoA. The observed stability of this compound may allow extended purification processes that will permit the study of its effect on luciferase bioluminescent reaction

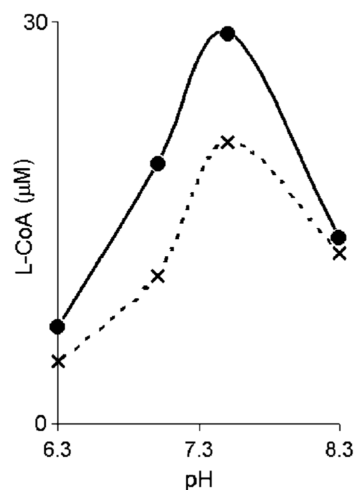

Figure 6. Effect of the pH on the luciferase catalyzed synthesis of L-CoA from L, ATP and CoA (●; continuous line) or from L-AMP and CoA (×; broken line).

and test the conflicting theories on the mechanism of the stimulating effect of CoA on light emission.[5,6,16,17]

Acknowledgements

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11. L (380 μmol) was dissolved in THF until total solubilization (about 100 mL). To this solution 800 μmol of carbonyldiimidazole in THF were added and the reaction mixture was left overnight. The solvent was evaporated off and the residue was dissolved in THF–H_20 (2:1). This solution was supplemented with 250 μmol of CoA dissolved in 25 mL of THF–H_20 (2:1); the pH of the reaction was adjusted to 7.0–7.5 with 0.5 M NaOH and the reaction mixture was left for about one day. THF was evaporated

off and the residual aqueous solution was acidified to pH 3–4 with the addition of small amounts of a cation exchange resin (Amberlite IR-120-H^+). The resin was removed by filtration, and the filtrate was extracted with diethyl ether (2×20 mL) to remove unreacted L. The aqueous phase and the dissolved residue of the organic phase (after evaporation of the ether) were analyzed by RP-HPLC. 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 (UV–vis) diode array detector. The eluent was a solution of 29% methanol, 2 mM phosphate buffer (pH 7.0), and the flow rate was set to 1 mL/min. The aqueous phase was lyophilized.

12. The purification of L-CoA present in the aqueous phase was performed by HPLC using a Supelco semi-preparative chromatographic column, the eluent was a solution of 35% methanol, phosphate buffer pH 7.0, with a flow rate of 2 mL/min.
13. The reaction mixture for the enzymatic synthesis of L-CoA contained in the final volume of 200 μL: 0.5 mM CoA, 60 μM L-AMP, 100 mM Hepes buffer (pH 7.8) and luciferase (0.08 mg protein/mL). At different times of incubation (0–10 min) 35 μL aliquots were withdrawn from the reaction mixtures and added to 35 μL of 10 mM EDTA in 66% methanol. Aliquots (50 μL) were injected, the flow rate was 0.6 mL/min and the eluent was a solution of 29% methanol (v/v) and 1 mM phosphate buffer (pH 7). These elution conditions were also used with chemically synthesized L-CoA (see Fig. 4).
14. In the assays studied by fluorescence the reaction mixtures contained in the final volume of 600 μL: 50 μL of partially purified L-CoA (estimated final concentrations of L-CoA 2.5 μM, L 0.13 μM in 2 mM phosphate buffer pH 7.0), 200 μM AMP, 200 μM PPi and luciferase (0.011; 0.022; 0.033 mg protein/mL). The reactions were initiated with the addition of luciferase and were performed in quartz cell in a Perkin–Elmer luminescence spectrometer LS-50 (excitation 340 nm; emission 550 nm). In the assays studied by RP-HPLC the reaction mixtures contained in the final volume of 100 μL: 10 μL of partially purified L-CoA (estimated concentration of 140 μM L-CoA and 7 μM L); 1 mM AMP, 1 mM PPi, 100 mM Hepes (pH 7.8) and luciferase (0.13 mg protein/mL). At different times of incubation (0–60 min) 20 μL aliquots were withdrawn and added to 40 μL of 10 mM EDTA in 66% methanol. The elution conditions were the same as referred above.
15. The reaction for the enzymatic synthesis of L-CoA from ATP, L and CoA in different pH media contained the following in a total volume of 60 μL: 30 μM L, 500 μM ATP, 2 mM $MgCl_2$, 100 mM buffer (MES pH = 6.3 or HEPES pH = 7.0, 7.5 or 8.3), PPase 1 U/mL and luciferase (0.035 mg protein/mL). In the case of the synthesis of L-CoA from L-AMP (30 μM) and CoA the reactions mixtures did not contain ATP, L, $MgCl_2$ and PPase. After 5 min (synthesis from ATP, L and CoA) or 30 s (synthesis from L-AMP and CoA) of incubation, 15 μL aliquots were withdrawn from the reaction mixtures, added to 66% methanol and analyzed by RP-HPLC as referred above.

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