Enzymes

Synthesis of Luciferyl Coenzyme A: A Bioluminescent Substrate for Firefly Luciferase in the Presence of AMP**

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Firefly luciferase (EC 1.13.12.7) is an oxidoreductase that catalyzes the bioluminescent reaction of luciferin (LH_2), ATP, and oxygen [Eq. (1) and (2)].[1,2] Research has been focused

$$LH_2 + ATP\text{-}Mg \rightleftharpoons LH_2\text{-}AMP + PPi \quad (1)$$

$$LH_2\text{-}AMP + O_2 \rightarrow Oxyluciferin + AMP + CO_2 + h\nu \quad (2)$$

on the clarification of the mechanism behind light emission, and as a consequence of its high quantum yield,[3] this reaction was defined as a model for the optimization of chemiluminescence systems.[4] The applications of firefly luciferase reactions range from ATP determination to the standard use of the *luc* gene in molecular biology.[5–7]

McElroy et al.[8] described the similarities between the mechanism of firefly luciferase reactions [Eq. (1)–(4)] and

$$LH_2\text{-}AMP + CoA \rightleftharpoons LH_2\text{-}CoA + AMP \quad (3)$$

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

those of the acyl-CoA synthetases [Eq. (5) and (6)]. This

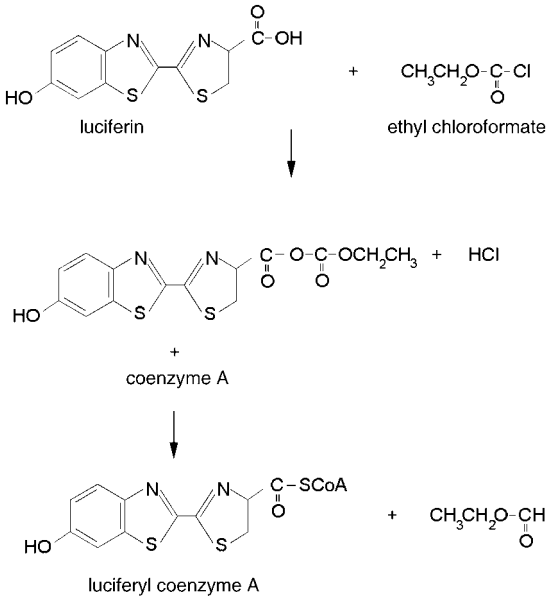
$$fatty\ acid + ATP\text{-}Mg \rightleftharpoons acyl\text{-}AMP + PPi \quad (5)$$

$$acyl\text{-}AMP + CoA \rightleftharpoons acyl\text{-}CoA + AMP \quad (6)$$

relationship was supported by the luciferase formation of the intermediate luciferyl adenylate (LH_2-AMP) and the production of dehydroluciferyl coenzyme A (L-CoA) from dehydroluciferyl adenylate (L-AMP); the latter compound is an oxidation product of LH_2-AMP (Supporting Information). Since then, several experimental observations have provided support for the McElroy hypothesis: the synthesis of dinucleoside polyphosphates (observed in acyl-CoA synthetases),[9] the high primary degree of sequence identity with rat long chain acyl-CoA synthetase (35.8%),[10] and recently the ability of luciferase to catalyze directly acyl-CoA formation [Eq. (5) and (6)].[11]

Herein we report the chemical synthesis and characterization of a new product in the firefly luciferase mechanism, namely, luciferyl coenzyme A (LH_2-CoA). The luciferase-catalyzed formation of LH_2-CoA from LH_2-AMP and coenzyme A [Eq. (3)] in anoxic media was recently advanced.[12] In that work, however, the unequivocal characterization of the enzymatically produced compound was not performed owing to the low catalytic activity that made the isolation of the formed compound impractical.

The chemical synthesis methodology employed in this work to obtain LH_2-CoA was based on the procedures of Simon and Shemin and of Wieland and Rueff (Scheme 1),[13,14] in which an acid anhydride is allowed to



Scheme 1. Chemical synthesis of LH_2-CoA.

react with a thiol in cold aqueous solution. As luciferin anhydride is not readily available, a mixed anhydride obtained from luciferin and ethyl chloroformate was used (Scheme 1).[15] By using this synthetic scheme we synthesized LH_2-CoA and confirmed its identity as the firefly luciferase product, which has already been detected.[12]

The formation of acyl-CoA catalyzed by acyl-CoA synthetases is readily reversible,[16] and the same was observed in the case of the luciferase-catalyzed formation of L-CoA.[2,17] Both the reverse and direct reactions occur through the same adenylated intermediates: L-AMP and acyl-AMP for luciferase and acyl-CoA synthetases, respectively [Eq. (4) and (6)]. In the case of LH_2-CoA, the corresponding adenylate is LH_2-AMP, which is also the direct intermediate in the bioluminescent reaction [Eq. (2) and (3)].

If Equation (3) could be reversed, luciferase would catalyze the conversion of AMP and LH_2-CoA into LH_2-AMP. This adenylate in the presence of oxygen would be oxidized to oxyluciferin, the light emitter. This hypothesis was

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confirmed by the observed light production when LH_2-CoA and AMP were mixed in the presence of firefly luciferase (Figure 1). No light was emitted in the controls performed with LH_2-CoA and without AMP injection, thus confirming that the conversion of this compound into LH_2-AMP is essential for light emission.

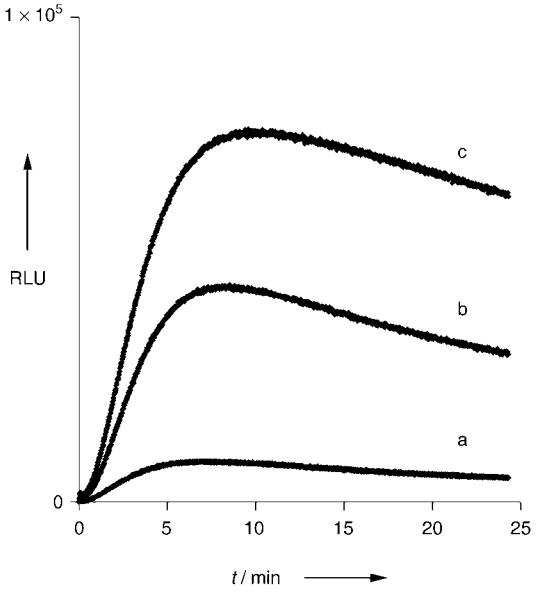

Figure 1. Bioluminescence from LH_2-CoA and AMP. The reaction mixtures contained purified and desalted LH_2-CoA (4 mM), EDTA (10 mM), hepes pH 7.5 (50 mM), and luciferase (0.04 mg protein mL^{-1}) in a final volume of 100 μL. The reactions were initiated with the injection of AMP. Curves a, b and c correspond to 62.5, 250, and 500 μM AMP, respectively (final concentrations).

The synthesized LH_2-CoA was purified but still contained traces of LH_2 as contaminant. In the presence of LH_2 any residual ATP would generate light through the direct adenylation of LH_2 [Eq. (1) and (2)]. To confirm that bioluminescence resulted from LH_2-CoA and AMP, the assays were supplemented with EDTA (10 mM). The presence of EDTA in this relatively high concentration prevented any possibility of direct adenylation because this process is Mg^{2+} dependent [Eq. (1)].[1,2]

The light emitted during the assay time was directly proportional to the concentration of AMP (up to 500 μM, Figure 2). Under our experimental conditions, that is, in the presence of 10 mM EDTA, the response obtained with ATP (5 mM, the highest AMP concentration used) was negligible (less than 5%).

The kinetic profile of LH_2-CoA + AMP bioluminescence (Figure 1) was markedly different from the flash profile of the LH_2 + ATP-Mg^{2+} (classic) bioluminescent reaction. In LH_2-CoA + AMP bioluminescence the rate of light production increased with incubation time, reaching a maximum at 10–20 min. Slow rise time patterns have already been described for LH_2-AMP analogues and luciferase mutants.[18]

As we have worked with native enzyme, and LH_2-AMP is a common intermediate in LH_2-CoA + AMP and classic bioluminescence, we deduce that the light profile obtained should be a consequence of the slow kinetics of the reaction of formation of LH_2-AMP from LH_2-CoA and AMP [Eq. (3)].

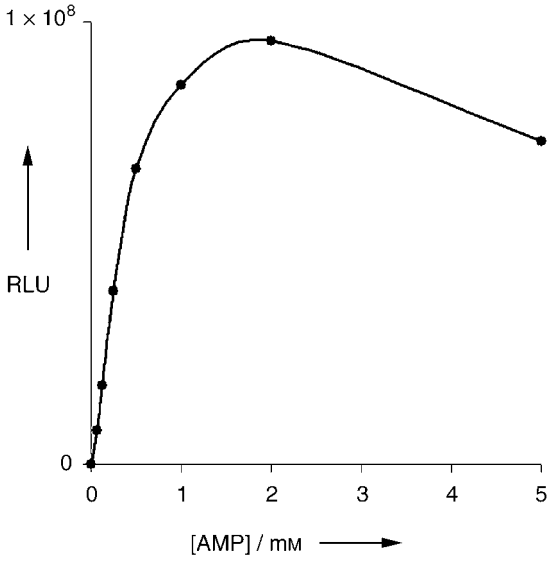

Figure 2. Total light output during the assay time (25 min) as a function of added AMP.

We had previously observed that the conversion of LH_2-AMP into LH_2-CoA is a relatively slow process.[12]

The identification of a new bioluminescent substrate for luciferase opens perspectives for the development of new bioanalytical methodologies for AMP detection. Moreover, the results presented herein are strong evidence for an evolutionary relationship between luciferase and acyl-CoA synthetases. From Equations (1)–(6) it is clear that if we exclude the light-production reaction [Eq. (2)], the mechanisms presented by firefly luciferase are in all ways similar to those of this class of enzymes.

Experimental Section

A stock solution of commercial luciferase (L8506) was prepared by dissolving the lyophilized powder (15 mg lyophilisate mL^{-1}) in hepes pH 7.5 (500 mM; 15 mg lyophilisate mL^{-1}) and stored at −20 °C. A stock solution of EDTA was prepared in hepes pH 7.5 (500 mM). Firefly luciferin, firefly luciferase, AMP, ATP, coenzyme A, EDTA, and hepes were purchased from Sigma, ethyl chloroformate from Aldrich, and triethylamine from Fluka.

Identification of enzyme-produced LH_2-CoA: The reaction mixture (15 μL) for the luciferase (Lase) synthesis of LH_2-CoA used ATP (1 mM), LH_2 (30 μM), coenzyme A (1 mM), $MgCl_2$ (2 mM), hepes pH 7.5 (100 mM), and Lase (1 mg protein mL^{-1}). All the solutions were prepared and kept under N_2. The reaction was initiated by the addition of Lase and was performed at ambient temperature. After 30 min of incubation, the enzyme reaction was stopped by the addition of aqueous methanol (15 μL; 66% v/v), centrifuged (13400 rpm, 2 min), and the supernatant was analyzed by reversed-phase HPLC. The chromatographic system consisted of a HP-1100 isocratic pump, a Rheodyne manual injection valve, a Chromolith C18 (Merck) column, and a Unicam Crystal 250 UV/Vis diode array detector. The eluent was an aqueous solution of 20% methanol, 4 mM phosphate buffer (pH 7.0), and the flux rate was set to 1 mL min^{-1}. Although a different order of elution could be obtained by using other phosphate buffer concentrations, under the above conditions LH_2-CoA (12 min) eluted after LH_2 (7 min). The spectra obtained for LH_2-CoA was similar to that reported previously,[12] with maxima at 262 and 340 nm ($A_{260}/A_{340} = 1.15:1$); see Supporting Information.

Chemical synthesis of luciferyl coenzyme A: The synthesis of luciferyl coenzyme A was based on previously reported methods for acyl-CoA thioester preparation.[13–15] LH_2 (50 mg, 0.178 mmol) in THF (15 mL) was mixed with triethylamine (25 μL, 0.178 mmol) at 0 °C. Ethyl chloroformate (17 μL, 0.178 mmol) was then added, and the mixture was left at 0 °C for 30 min. The volume of the reaction mixture was reduced to a half under a stream of N_2, and a mixture of coenzyme A (68.7 mg; 0.09 mmol) and $NaHCO_3$ (20 mg) in THF/H_2O (2:1; 10 mL) was added. The pH value was maintained at 7.5–8. After stirring for 30 minutes the reaction mixture was analyzed by reversed-phase HPLC as described for the identification of enzyme-produced LH_2-CoA. This system allowed the resolution of reactants and products, and the reaction was monitored at regular time intervals until no more LH_2-CoA formation was observed (120 min).

The retention time observed for the chemically synthesized compound was the same as that observed with enzyme-produced LH_2-CoA (Supporting Information). The THF was evaporated, and the aqueous solution was acidified to pH 3–4 by 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 unconverted LH_2. The aqueous phase and organic phase were analyzed by reversed-phase HPLC, and as expected, no LH_2-CoA was detected in the diethyl ether.

The aqueous phase was lyophilized and a green fluorescent powder was obtained. LH_2-CoA was purified by reversed-phase HPLC by using a Supelco semipreparative column (LC-18, 25 cm × 10 mm, 5 μm) with a solution of methanol (40%) and phosphate buffer (11–15 mM; pH 7.0) as eluent; the flux rate was set to 3 mL min^{-1}. MALDI TOF analysis of purified LH_2-CoA was performed at RIAIDT, Unidad de Espectroscopia de Masas, Santiago de Compostela, Spain, and confirmed the calculated mass for $C_{32}H_{42}N_9O_{18}P_3S_3$, $M_w = 1029.842$ g mol^{-1} (Supporting Information). Prior to MS analysis and bioluminescence assays, LH_2-CoA solutions were desalted by using a ZipTip from Millipore; the phosphate content on the treated sample was verified by a variation of the molybdate method.[19] Purified LH_2-CoA stored at −20 °C was stable during the period of the bioluminescent assays (2 weeks), as confirmed by reversed-phase HPLC.

Bioluminescence from LH_2-CoA and AMP: The bioluminescence tests were performed in a homemade luminometer by using a Hammamatsu HCL35 photomultiplier tube. The reaction mixtures contained purified and desalted LH_2-CoA (4 μM), EDTA (10 mM), hepes pH 7.5 (50 mM), and luciferase (0.04 mg protein mL^{-1}); final volume: 100 μL. The reactions were initiated with the injection of AMP. The assays were carried out in duplicate, and control assays with water and ATP (5 mM, with and without EDTA (10 mM)) were also performed. Light was measured for 25 minutes with integrations at 1-s intervals.

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