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PAPER

Kinetics of inhibition of firefly luciferase by dehydroluciferyl-coenzyme A, dehydroluciferin and L-luciferin†

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The inhibition mechanisms of the firefly luciferase (Luc) by three of the most important inhibitors of the reactions catalysed by Luc, dehydroluciferyl-coenzyme A (L-CoA), dehydroluciferin (L) and L-luciferin (L-LH_2) were investigated. Light production in the presence and absence of these inhibitors (0.5 to 2 μM) has been measured in 50 mM Hepes buffer (pH = 7.5), 10 nM Luc, 250 μM ATP and D-luciferin (D-LH_2, from 3.75 up to 120 μM). Nonlinear regression analysis with the appropriate kinetic models (Henri–Michaelis–Menten and William–Morrison equations) reveals that L-CoA is a non-competitive inhibitor of Luc ($K_i = 0.88 \pm 0.03$ μM), L is a tight-binding uncompetitive inhibitor ($K_i = 0.00490 \pm 0.00009$ μM) and L-LH_2 acts as a mixed-type non-competitive-uncompetitive inhibitor ($K_i = 0.68 \pm 0.14$ μM and $\alpha K_i = 0.34 \pm 0.16$ μM). The K_m values obtained for L-CoA, L and L-LH_2 were 16.1 ± 1.0, 16.6 ± 2.3 and 14.4 ± 0.96 μM, respectively. L and L-LH_2 are strong inhibitors of Luc, which may indicate an important role for these compounds in Luc characteristic flash profile. L-CoA K_i supports the conclusion that CoA can stimulate the light emission reaction by provoking the formation of a weaker inhibitor.

Introduction

Firefly luciferase (EC 1.13.12.7) is an enzyme that catalyzes the oxidation of firefly luciferin (LH_2), giving rise to light in a two-step reaction:[1,2] the first step involves the formation, from D-LH_2 and adenosine-5′-triphosphate (ATP), of an adenylyl intermediate (D-LH_2-AMP) (eqn (1)). The second step consists on the oxidation of D-LH_2-AMP, and the release of adenosine-5′-monophosphate (AMP), CO_2 and oxyluciferin ($OxyLH_2$) (eqn (2)). The light emitter is formed in an excited singlet state S_1, decaying to the ground state with the emission of visible light (550–570 nm). This system is known for its efficiency when compared with chemiluminescence. For many years the efficiency of this reaction was thought to be of 88%,[3,4] but a recent work performed by Niwa *et al.*[5] determined the quantum yield of several beetle luciferase to be 45–61%. Despite the sharp decrease, these new values still strongly support the study and the development of practical applications for this bioluminescence system. Currently, it has gained numerous bioanalytical, biomedical and pharmaceutical applications, among others. More specifically, it is involved in the analytical determination of ATP, in microbial detection, imunoassays, bioimaging, biosensing and is used as a gene reporter.[1,2,6–12]

$$\text{Luc} + LH_2 + \text{ATP} \rightleftharpoons \text{Luc}{\cdot}LH_2\text{–AMP} + PP_i \quad (1)$$

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

Due to this broad range of applications, it is crucial to have a deep knowledge of the inhibitors of Luc and their inhibition parameters. These compounds may have a non-negligible effect on the bioluminescence reaction and can lead to erroneous results in Luc-catalyzed applications, as for example in reporter-gene assays. The current knowledge of Luc inhibition was recently reviewed by one of the present authors.[2]

The *in vitro* emission of light follows, at relatively high substrate concentration, a flash pattern, which starts with an initial flash that quickly decays to a low basal level. This characteristic of light emission is caused by an accumulation of inhibitory products.[1,2,6] Two of the most well characterized inhibitors of the bioluminescence reaction are $OxyLH_2$ ($K_i = 0.50 \pm 0.03$ μM), the reaction product, and dehydroluciferyl-adenylate (L-AMP).[2,13] L-AMP results from the oxidation of luciferyl-adenylate (LH_2-AMP) in a dark reaction also catalyzed by Luc, and acts as fast tight-binding competitive inhibitor ($K_i = 3.8 \pm 0.7$ nM) being responsible for the typical flash profile.[13] Besides its role as light-production inhibitor, this LH_2-AMP derivative can be used by Luc as a substrate, in another dark reactions:

$$\text{Luc}{\cdot}\text{L–AMP} + \text{CoA} \rightleftharpoons \text{Luc} + \text{L–CoA} + \text{AMP} \quad (3)$$

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$$\text{Luc·L–AMP} + PP_i \rightarrow \text{Luc} + \text{L} + \text{ATP} \quad (4)$$

The reaction of L-AMP with coenzyme A (CoA) and inorganic pyrophosphate (PP_i) has an antagonizing effect on light inhibition, as it results in dehydroluciferyl-coenzyme A (L-CoA) and dehydroluciferin (L), which experimental evidence indicates that these compounds are weaker Luc inhibitors than L-AMP.[14–16] As L results from the oxidation of LH_2 and ATP, through the formation of L-AMP and its posterior pyrophosphorolysis,[15,16] it was initially considered as the light emitter, instead of $OxyLH_2$.[17] Its chemical synthesis[18] permitted the clarification of L role in the bioluminescence reaction, and its incubation with Luc and ATP-Mg^{2+} characterized it as a Luc inhibitor.[14] Some groups[19–22] hypothesized a competitive mechanism of inhibition for L with respect to D-LH_2 with a K_i of about 0.10–1.20 μM.[23] However there is still no evidence to support any inhibition mechanism for L.

CoA does not participate in the "classic" bioluminescence reaction, but due to its light-production stimulating effect[14] is now added to Luc commercial assays. It is thought that this effect is achieved by the conversion of L-AMP into L-CoA, a supposed much weaker inhibitor. However, no study to date has ever elucidated its inhibition constant and mechanism.

The *in vitro* synthesis of LH_2 leads to two enantiomers, D and L, according to the cysteine isomer used.[1,6,8] D-LH_2 is regarded as the only isomer to naturally produce light,[1,6] while the L-isomer is an inhibitor.[24,25] Lembert[25] studied its inhibitory mechanism and suggested that L-LH_2 acts as a competitive inhibitor with a K_i of 3–4 μM. However, and despite Lembert's important contribution to the existing literature, more accurate values are needed in order to clarify the importance of this isomer in this bioluminescent light profile.

In the current study we provide a detailed kinetic model of the inhibition exerted by these three compounds by measuring the light production in their presence and absence. The results obtained in this work suggest an important role for L and L-LH_2 in Luc flash profile due to their strong inhibitory character. The discovery that L-CoA is indeed a weaker inhibitor than L-AMP further supports the thiolytic mechanism regarding CoA stimulating effect on light production.

Experimental

Materials

A stock solution of commercial Luc (Sigma; L9506) was prepared by dissolving the lyophilized powder in Hepes buffer 0.5 M, pH 7.5 (15 mg lyophilisate per mL; 60 μM Luc) and stored in small aliquots at −20 °C to prevent self-degradation. Its concentration was confirmed by UV spectroscopy at 278 nm using the extinction coefficient of 45 560 M^{-1} cm^{-1}.[26] D-LH_2, ATP and Hepes were also purchased from Sigma.

L and L-LH_2 were chemically synthesized and purified as described previously.[18,27–30] The chemical synthesis of L-CoA was based on the chemical synthesis of LH_2-CoA performed by Fraga *et al.*[31] D-LH_2 (50 mg) in THF (2 mL) was mixed with triethylamine (0.75 mL) and ethyl chloroformate (0.51 mL). The reaction was left at 25 °C for 5 h, and the reaction mixture volume was evaporated under a stream of N_2. The addition of a mixture of CoA (81 mg) and dimethyl sulfoxide (2 × 3 mL) to the reaction mixture, and posterior contact with atmospheric oxygen resulted on the formation of L-CoA. The initial objective for this synthesis was the obtaining of D-LH_2-CoA, but contact with oxygen lead to the formation of this oxidized derivative. Its purity was assessed by LC-MS and RP-HPLC, and the synthesised compounds have the following percentage of purity: L-CoA 88.4%, L 95.0% and L-LH_2 90.5% (ESI†). Taking into consideration the percentage of purity of the synthesised L-CoA, L and L-LH_2, calculated as indicated in the ESI, they were weighed accurately and dissolved in water and Hepes 0.5 M (pH 7.5), respectively, to a final concentration of 250 μM, 167 μM and 208 μM respectively.

Luc-catalysed light production assays

All the enzyme reactions took place at ambient temperature (24–27 °C) and were performed at least in triplicate. The bioluminescence tests were performed in a homemade luminometer using a Hamamatsu HC135-01 photomultiplier tube. All light reactions were carried out in 50 mM Hepes buffer and pH 7.5. The reaction was initiated by the injection of D-LH_2 (3.75–120 μM – volumes of 50 μL) into a transparent assay tube, by simple reagent mixing, containing a mixture of ATP (250 μM), $MgCl_2$ (2 mM) and Luc (10 nM). In some experiments, this latter mixture was supplemented with L, L-LH_2 or L-CoA (in the concentration range between 0.5 and 2 μM). All the indicated concentrations refer to the final volume of 200 μL. The light was integrated and recorded in 0.1 s intervals. L, L-LH_2 and L-CoA solutions were protected from light at all time by covering the tubes with aluminium foil.

The steady-state initial velocities were determined by incubating Luc with serial dilutions of the inhibitors for 3 min at room temperature, followed by the addition of D-LH_2. The reaction was allowed to continue for 3 min. Reaction rates were determined from the increase in light production over the first milliseconds after the start of the reaction in the linear part of the flash profile.

Inhibition models and data analysis

Analysis of the steady-state kinetics of the inhibition of Luc by L-CoA and L-LH_2. The apparent Michaelis constant (K_m^{app}) and the apparent maximum velocity (V_{max}^{app}) values were determined from the nonlinear least-squares fit of the Henri–Michaelis–Menten equation:[32]

$$v = \frac{V_{max}^{app}[S]}{K_m^{app} + [S]} \quad (5)$$

Dissociation constant values (K_i) for L-CoA were estimated from the nonlinear least-squares best fit of the non-competitive inhibition model:[32]

$$v = \frac{V_{max}}{1 + \frac{[I]}{K_i}} \frac{[S]}{[S] + K_m} \quad (6)$$

where K_m is the Michaelis constant and V_{max} the maximum velocity.

Dissociation constant values (K_i) for L-LH_2 were estimated from the nonlinear least-squares best fit of the mixed-type inhibition model:[33]

$$v = \frac{\frac{V_{max}}{1+\frac{[I]}{\alpha K_i}}[S]}{[S] + \frac{K_m\left(1+\frac{[I]}{K_i}\right)}{1+\frac{[I]}{\alpha K_i}}} \quad (7)$$

where K_m is the Michaelis constant and V_{max} the maximum velocity.

Analysis of the steady-state kinetics of the inhibition of Luc by L. The K_i^{app} values for L were determined according to the Williams and Morrison equation:[32]

$$\frac{v}{v_0} = \frac{[E]-[I]-K_i^{app}+\sqrt{([E]-[I]-K_i^{app})^2+4[E]K_i^{app}}}{2[E]} \quad (8)$$

where [E] is the active enzyme concentration, [I] is the total inhibitor concentrations, K_i^{app} is the overall dissociation constant, v is the initial reaction velocity at inhibitor concentration [I], and v_0 is the initial velocity in the absence of the inhibitor. If the inhibitor presents different affinity for the enzyme alone and for the complex enzyme-substrate, K_i^{app} is related to the dissociation constant of the inhibitor (K_i) by the equation:[32]

$$K_i^{app} = \frac{[S]+K_m}{\frac{K_m}{K_i}+\frac{[S]}{\alpha K_i}} \quad (9)$$

where [S] is the competing substrate concentration, K_i the inhibition constant with respect to the unbound enzyme and αK_i the inhibition enzyme referring to the enzyme–substrate complex.

Data analysis

All data were analyzed by nonlinear regression analysis using eqn (5) to (9) with Graphpad software package (GraphPad Prism 5.01 for Windows).

Results and discussion

L-CoA inhibition kinetics

Fig. 1 shows the first three seconds of typical Luc flash profiles. The flash profile in the presence of L-CoA shows a considerable decrease in the maximum of light intensity and in the initial velocity under steady-state conditions, confirming its inhibitory properties.

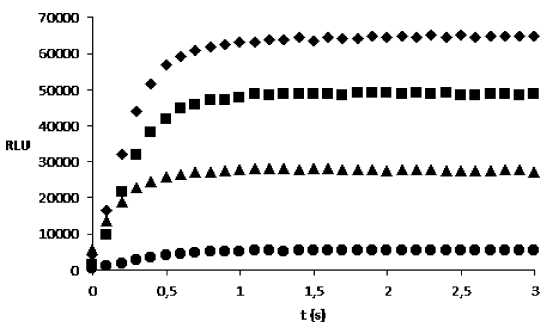

Fig. 1 Light emission data from standard bioluminescence activity assays of Luc performed in the presence of (■) L-CoA 0.5 μM, (▲) L-LH_2 0.5 μM, (●) L 0.5 μM and in the absence (◆) of inhibitors. D-LH_2 15 μM was injected (at t = 0 s) into mixtures containing ATP 250 μM, Luc 10 nM and Mg^{2+} 2 mM in Hepes buffer (pH = 7.5) pre-incubated with inhibitor.

Table 1 Kinetic parameters for the inhibition of Luc by L-CoA

[L-CoA]/μM	K_m^{app}/μM	$V_{max}^{app}/\times10^{-5}$ RLU s^{-1}
0	15 ± 1	2.29 ± 0.3
0.50	18 ± 1	1.56 ± 0.12
1.0	17 ± 2	1.17 ± 0.15
1.5	18 ± 2	0.84 ± 0.1
2.0	21 ± 2	0.70 ± 0.06
K_i/μM	0.88 ± 0.03	
$V_{max}/\times10^{-5}$ RLU s^{-1}	2.34 ± 0.04	
K_m/μM	16.1 ± 1.0	

L-CoA was found to inhibit Luc in a non-competitive fashion with a K_i of 0.88 ± 0.03 μM (Table 1). This value was determined from a nonlinear fit of eqn (6) to steady-state initial velocities obtained with several concentrations of D-LH_2, L-CoA and saturating concentrations of ATP (Fig. 2a). This inhibition followed a characteristic hyperbolic Henri–Michaelis–Menten pattern.

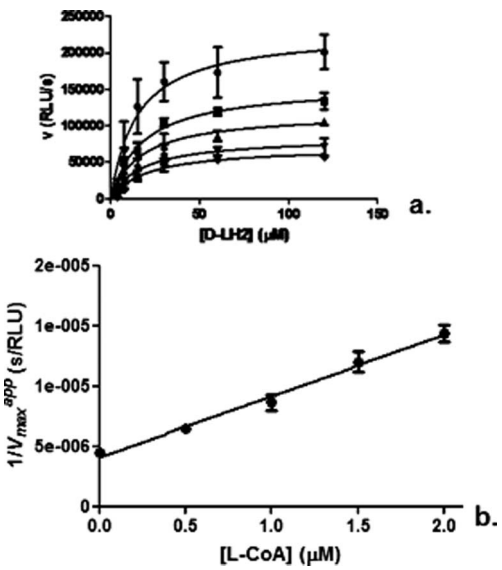

Fig. 2 (a) The dependence of the initial velocity on the concentration of D-LH_2 at different concentrations of L-CoA:(■) –0.5 μM, (▲) –1.0 μM, (▼) –1.5 μM, (◆) –2.0 μM, and without inhibitor (●). The lines represent a least squares best fit of the Henri–Michaelis–Menten equation to the data. The fit parameters, K_m^{app} and V_{max}^{app} are represented on Table 1. (b) The dependence of $1/V_{max}^{app}$ on L-CoA concentration. All experiments were performed using 50 mM Hepes buffer (pH 7.5) at ambient temperature as described in experimental. The Luc concentration was 10 nM.

The secondary plot (Fig. 2b; $1/V_{max}^{app}$ as a function of inhibitor concentration) shows that an increase in L-CoA concentration resulted in a linear decrease of V_{max}^{app}. These results indicate that L-CoA is a non-competitive inhibitor towards D-LH_2.

Lineweaver–Burk plot (Fig. 3a) exhibit a linear trend typical of a non-competitive inhibitor, with the same intercept on the 1/[D-LH_2] axis, further underlining the non-competitive behaviour of this compound. These results demonstrate that L-CoA binds with identical affinity to the free enzyme and the enzyme–substrate complex, and that this binding impairs the light-production

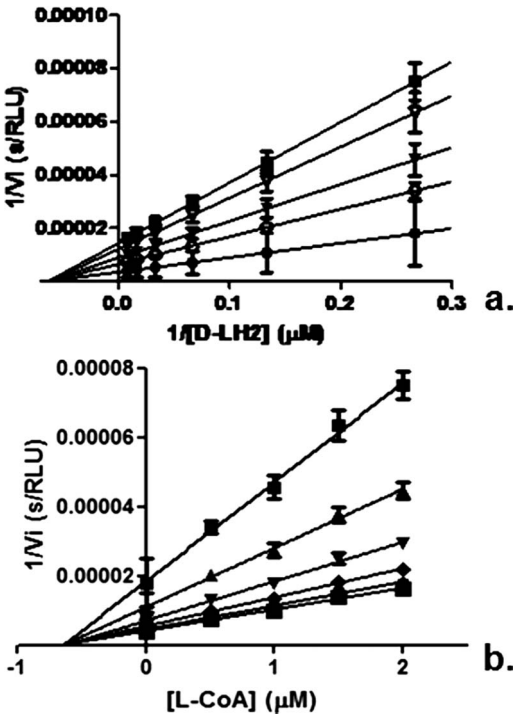

Fig. 3 (a) Lineweaver–Burk plot of inhibition of Luc by L-CoA with respect to D-LH_2. L-CoA concentrations: (■) 2.0, (▽) 1.5, (▼) 1.0, (○) 0.5 and (●) 0 μM. (b) Dixon plot of the same data used in plot (a). D-LH_2 concentration: (■) 120, (●) 60, (◆) 30, (▼) 15, (▲) 7.5 and (■) 3.75 μM.

reaction. It is known that Luc has acyl-CoA synthetase activity, being part of the acyl-adenylate/thioester-forming superfamily of enzymes.[24,34] The members of this family catalyze a two-step reaction: first exist an initial adenylation of a carboxylate, forming a adenylate intermediate; in the second step, this intermediate is commonly involved in the formation of a thioester. Recent experimental evidence indicate that these enzymes use a 140° domain rotation to present opposing faces of a dynamic C-terminal domain to active site for the different partial reactions.[34] On the contrary, structural analysis of the bioluminescence reaction indicates that there are only minor conformational changes during this catalysis, and that the Luc active site remains in the conformation corresponding to the adenylation step during light production.[35] Analysis with Luc complexed with ATP, an adenylyl analogue and AMP and OxyLH_2, generating a series of "snapshots" of the bioluminescence reaction, demonstrated that the different complexes are in similar conformations. Thus, if we have an enzyme complexed with one of substrates, the intermediate and the two products in the same conformation, it is reasonable to assume that this reaction does not need large conformational changes to occur. So, there are indications that L-CoA can bind to different conformations of Luc, corresponding to the absence or presence of D-LH_2, causing a dynamic domain rotation that leads to the more stable Luc conformation for CoA-thioesters. As there are no major changes in Luc structure during the bioluminescence reaction, the stabilization of the active site should be essential to the phenomenom and so the change of conformation induced by L-CoA could hinder the binding of D-LH_2 to the enzyme, accounting for the observed inhibition. Alternatively, there are evidences that contradict the structural analysis and state that the bioluminescence reaction does suffer a large conformation change during its two-step reaction. A mutagenesis work performed by Branchini *et al.*[36] indicated that two different and opposite C-domain lysine residues are each one essential to only one of the two-steps of this reaction. Thus, this indicates the occurrence of a large C-domain rotation when the product of the adeynlation step becomes the substrate for the second step of light-production. Thus, taking these data in consideration L-CoA could inhibit the bioluminescence reaction, not by hindering D-LH_2 binding site, but by having the ability to bind to the two conformations of Luc, and so, compete with the substrates of the two-steps. However, it should be said that the major Luc mutants used in this study showed bioluminescence spectra different from the wild-type, indicating that the mutations produced structures that could be too different to be compared with the wild-type. Moreover, the role of the two lysines was assessed by calculating the steady-state constants of LH_2-AMP and L. Although there is evidence that support the use of LH_2-AMP,[35] our work demonstrates that L do not bind at the same site of D-LH_2, and so the adenylation of these two compounds may not occur at the same conditions. Thus, more data is needed about Luc conformation during the bioluminescence reaction prior to more conclusive explanations.

Also, the relatively weak inhibitory character of L-CoA when in comparison with L-AMP (K_i = 3.8 ± 0.7 nM)[13] is in good agreement with the experimental work that indicates that the stimulating effect exerted by CoA on the bioluminescence reaction is caused by its interaction with L-AMP and subsequent formation of L-CoA.[14]

L inhibition kinetics

The flash profile in the presence of L (Fig. 1) shows a sharp decrease in the maximum of light intensity and in the initial velocity under steady-state conditions. This decrease is more pronounced than for L-CoA and L-LH_2, illustrating the strong inhibitory potency of this LH_2 derivative. Moreover, this inhibitory power greatly resembles the inhibitory profile of L-AMP, which only differs from the bioluminescence reaction adenylyl intermediate by its dehydroluciferin moiety. This evidence indicates a tight-binding inhibition mechanism for L.

The K_i^{app} values were estimated by nonlinear regression using the Morrison equation which accounts for the tight-binding inhibition (eqn (8) and Fig. 4) and are shown in Table 2. The inhibition mechanism of L was determined by plotting the IC_{50} value, concerning each substrate concentration, as a function of D-LH_2 concentration (Fig. 5a). The obtained graphical representation is typical of a non-competitive inhibition mechanism when $\alpha < 1$, also termed as mixed-type inhibition.[33] The true K_i and αK_i values were determined by non-linear fitting of the K_i^{app} values to eqn (9), as a function of D-LH_2 concentration (Fig. 5b). The values obtained were $K_i = \infty$ and $\alpha K_i = 0.00490 \pm 0.00009$ μM, thus indicating L basically acts as an uncompetitive inhibitor towards D-LH_2.

An uncompetitive inhibition mechanism suggest that L binds only to the enzyme–substrate complex and do not compete with the substrate for the active site, suggesting the presence of a different binding site. This may sound strange, considering the obvious structural similarities of L with D-LH_2, which indicate that even if there was a presence of a new binding site during the

Table 2 Kinetic parameters for the inhibition of Luc by L

[L]/μM	K_i^{app}/μM
120	0.00070 ± 0.00001
60.0	0.0045 ± 0.0004
30.0	0.0073 ± 0.0006
15.0	0.0074 ± 0.0006
7.5	0.0100 ± 0.0023
3.75	0.0327 ± 0.0020
αK_i/μM	0.00490 ± 0.00009
K_m	16.6 ± 2.3

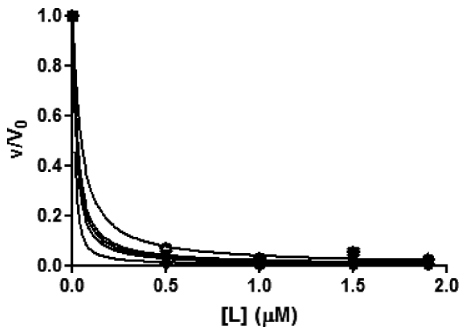

Fig. 4 Plot of fractional velocity as a function of L concentration at different D-LH_2 concentrations. The inhibition assays were conducted with D-LH_2: (●) 120 μM, (■) 60 μM, (▲) 30 μM, (▼) 15 μM, (◆) 7.5 μM, (○) 3.75 μM and ATP 250 μM in 50 mM Hepes buffer (pH 7.5) at room temperature. Enzyme concentration was 10 nM. L concentration was varied from 0 to 1.9 μM.

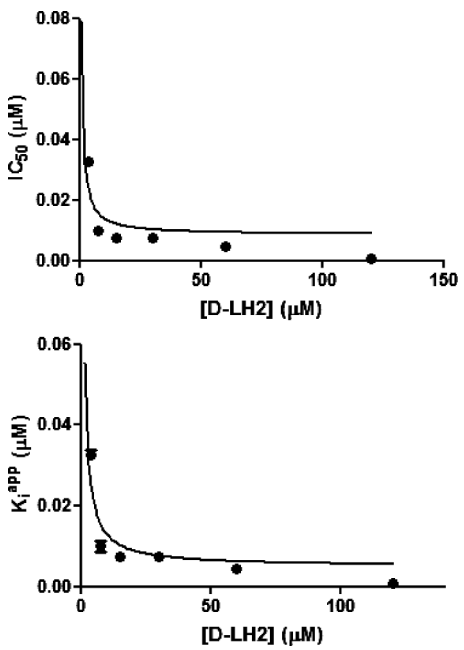

Fig. 5 (a) The effect of D-LH_2 concentration (3.75 to 120 μM) on the IC_{50} values. (b) Secondary plot of K_i^{app} as a function of D-LH_2. K_i^{app} values were obtained from the nonlinear fit of Fig. 4.

bioluminescence reaction, L should still bind to the active site. Furthermore, even if we consider the different orientations of the carboxylic group between these two molecules as a impediment for the binding of L, it is not clear as why $OxyLH_2$ (a competitive inhibitor towards D-LH_2[13] and a similar compound to L) does not bind in this supposed binding site. Another confusing fact regarding this subject is that L-AMP[13] and L-CoA, two L-derivatives, can bind to the free enzyme. So, the only explanation that appears to be logical is that L does not bind to a different binding site, but at the active site. The different affinities can be explained as it is known that the Luc active site suffers changes in its conformation during the bioluminescence reaction, changing from a more open to a more closed structure.[36] This more closed conformation possibly can better accommodate the different orientation of the carboxylic group of L. On the contrary, L-CoA and L-AMP can accommodate this carboxylic group in the free enzyme, possibly because their CoA and AMP moieties are so much bigger and have so many more points of interaction with the active site in their structure that they may "force" the positioning of the L moiety in the enzyme.

Alternatively, if we consider the work of Branchini *et al.*,[36] the mechanism of inhibition of L could be explained by the rotation of the C-domain of Luc during the bioluminescence reaction. This could indicate that L only binds to the conformation of the second-step of the bioluminescence reaction. However, there are some aspects of this mutagenesis work that need some clarification, as stated above in the present paper, that prevent its use in a more conclusive explanation. The study of the inhibition of L with respect to LH_2-AMP could also be of pivotal importance for the clarification of this topic.

L-LH_2 inhibition kinetics

The flash profile in the presence of L-LH_2 shows also a decrease in the maximum of light intensity and in the initial velocity under steady-state conditions, confirming its inhibitory properties. This decrease is intermediate between the observed for L-CoA and L, indicating that this inhibitor is stronger than L-CoA and weaker than L.

This inhibition followed a characteristic hyperbolic Henri–Michaelis–Menten pattern, revealing that the addition of L-LH_2 to the reaction mixtures causes a decrease in both V_{max}^{app} and K_m^{app}, indicating an uncompetitive mechanism for L-LH_2 (Fig. 6a and Table 3). However, the Lineweaver–Burk plot (Fig. 6b) shows that the slope is also affected, determining a mixed-type inhibition for L-LH_2 with a K_i of 0.68 ± 0.14 μM and a αK_i of 0.34 ± 0.16 μM. The values was determined from a nonlinear fit of eqn (7) to steady-state initial velocities obtained with several concentrations of D-LH_2, L-LH_2 and saturating concentrations of ATP (Fig. 5a).

Table 3 Kinetic parameters for the inhibition of Luc by L-LH_2

[L-LH_2]/μM	K_m^{app}/μM	V_{max}^{app}/×10^{-5} RLU s^{-1}
0	14.8 ± 1.9	1.027 ± 0.036
0.5	10.1 ± 1.4	0.484 ± 0.017
0.9	9.7 ± 1.0	0.282 ± 0.007
1.4	8.8 ± 0.6	0.174 ± 0.003
1.8	6.7 ± 1.1	0.084 ± 0.003
K_i/μM	0.68 ± 0.14	
α	0.50 ± 0.13	
αK_i/μM	0.34 ± 0.16	
K_m/μM	14.4 ± 0.96	

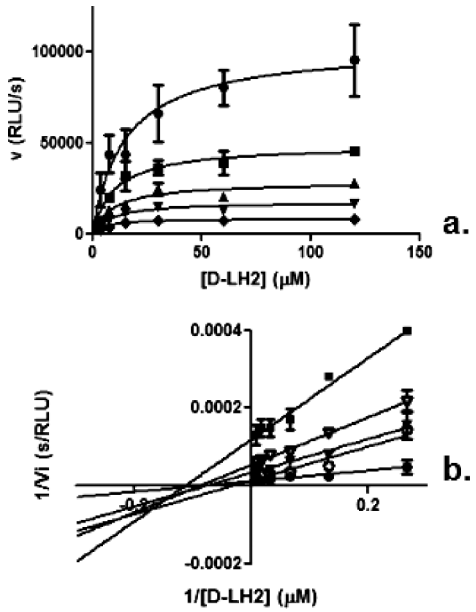

Fig. 6 The dependence of the initial velocity on the concentration of d-LH_2 at different concentrations of L-LH_2:(■) –0.5 μM, (▲) –0.9 μM, (▼) –1.4 μM, (◆) –1.8 μM, and without inhibitor (●). The lines represent a least squares best fit of the Henri–Michaelis–Menten equation to the data. The fit parameters, K_m^{app} and V_m^{app} are represented in Table 3. (b) Lineweaver–Burk plot of inhibition of Luc by L-LH_2 with respect to D-LH_2. L-LH_2 concentrations: (■) 1.8, (▽) 1.4, (▼) 0.9, (○) 0.5 and (●) 0 μM. All experiments were performed using 50 mM Hepes buffer (pH 7.5) at ambient temperature as described in experimental. The Luc concentration was 10 nM.

Lineweaver–Burk plot (Fig. 6b) exhibits a linear trend typical of a mixed-type inibitor, with different intercepts on the 1/[D-LH_2] and the $1/V_i$ axis, and different slopes. Furthermore, the plots cross to the left of the $1/V_i$ axis and below the 1/[D-LH_2] axis. This is typical of a form of mixed inhibition known as non-competitive-uncompetitive inhibition. So, these results show that L-LH_2 can bind both to the free enzyme and to the enzyme-substrate complex, but with different affinities. Thus, this type of inhibition can have some different explanations. First, we can consider a different binding site for L and D-LH_2, with the binding site conformation of the L-isomer becoming more favorable to their association during the bioluminescence reaction. We can also consider two binding sites for L-LH_2, one on the free enzyme and another that is formed during the bioluminescence reaction. However, it is unlikely that these two isomers bind only at different sites due to their obvious structural resemblance. Moreover, even if we consider that the opposite conformation of the carboxylic group that these molecules present can impair the binding in these different sites, we cannot explain why OxyLH_2 binds only to the D-LH_2 active site. As was referred in the introduction section, this LH_2-derivative is a competitive inhibitor with respect to D-LH_2,[13] and it is very similar to the (D/L)-isomers (presenting only a thiazolone moiety instead of a thiazoline-carboxylic acid one[1]). So, it should have the ability for binding wherever the isomers can. Its competitive inhibition mechanism towards D-LH_2 indicates that we are dealing with only one binding site for this type of molecule. Therefore, the more logical explanation for this situation is that the K_i value refers to a competitive inhibition of L-LH_2 towards its D-isomer. The existence of a lower αK_i value indicates that this molecule can still bind to the active site even after the formation of the binary complex Luc·LH_2-AMP, and that the active site is present in a more favorable conformation for their interaction. We know that L-LH_2 can be used by Luc to form L-LH_2-CoA to the contrary of D-LH_2,[24] that the Luc-catalyzed formation of CoA-thioesters is a more favorable reaction than the bioluminescence reaction[14] and that there are some changes in Luc conformations during the formation of the adenylyl intermediate.[35] So, it is reasonable to deduce that these changes result in a more favorable active site structure to the compounds that are involved in the formation of CoA-thioesters, as L-LH_2, thus resulting in higher affinities for the enzyme.

Tables 1, 2 and 3 show that the K_m values obtained for L-CoA (16.1 ± 1.0 μM), L (16.6 ± 2.3 μM) and L-LH_2 (14.4 ± 0.95 μM) agree with each other and the K_m reported by Branchini *et al.*[37] (15 μM).

Conclusions

The present work gives, for the first time, accurate K_i values and kinetic mechanisms for L-CoA, L and L-LH_2, indicating a possible importance of L and L-LH_2 for the characteristic *in vitro* flash profile of Luc.

The mechanism of inhibition by L was described for the first time in the present work. It acts as a tight-binding uncompetitive inhibitor of Luc with respect to substrate D-LH_2 (K_i = 4.90 ± 0.09 nM). In this type of inhibition the population of free soluble inhibitor is significantly depleted by the formation of the enzyme–inhibitor complex. This suggests that L binds almost irreversibly to the Luc active-site arresting the normal light-producing reactions. As the main difference of the structure of L, in comparison with D-LH_2, is the orientation of the carboxylic group, this fast tight-interaction with Luc could be due to the formation of a more stable hydrogen bond network, a sufficiently fast process to account to this inhibition mechanism. Since this inhibitor could be formed as a side product of Luc catalyzed light reactions, as indicated in eqn (4), and D-LH_2 could be oxidized into this compound our data indicate that L could have an important role in the typical flash profile.

Previous experimental observations have shown that the strong inhibition of Luc caused by L-AMP could be easily antagonised by addition of CoA, resulting in the formation of L-CoA. It is thought that the thiolytic reaction is much faster than the bioluminescent reaction and that the L-CoA is a much less powerful Luc inhibitor than L-AMP.[14] The mechanism of L-CoA inhibition was studied in this work. It was found to be a non-competitive inhibitor with respect to D-LH_2, less potent (K_i = 0.88 ± 0.03 μM) than L-AMP,[13] and both the magnitude of L-CoA inhibition and its kinetics of inhibition are in agreement with the stimulating effect exerted by CoA on the bioluminescence reaction.

L-LH_2 was found to be an mixed-type non-competitive-uncompetitive inhibitor with a K_i = 0.68 ± 0.14 μM and αK_i = 0.34 ± 0.16 μM. This value, along with its kinetics of inhibition, suggests that this inhibitor is not a key component of the fast decay of bioluminescence. However, the lack of knowledge of the conversion of D-LH_2 into L-LH_2 at bioluminescence reaction conditions, and its relevant K_i suggest that L-LH_2 could have an important role on the *in vitro* flash profile.

Abbreviations

AMP	Adenosine-5′-monophosphate
ATP	Adenosine-5′-triphosphate
L	Dehydroluciferin; L-AMP, dehydroluciferyl-adenylate
L-CoA	Dehydroluciferyl-CoA
Luc	Firefly luciferase; (D/L)-LH_2, firefly luciferin
LH_2-AMP	Luciferyl-adenylate; PP_i, inorganic pyrophosphate
RLU	Relative light units; $OxyLH_2$, oxyluciferin

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