Firefly luciferase has two nucleotide binding sites: effect of nucleoside monophosphate and CoA on the light-emission spectra

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A laboratory-made spectroluminometer was used to analyse the light emitted by firefly (*Photinus pyralis*) luciferase reacting with several nucleotide derivatives. The analysis of the light emission in the presence of ATP or dATP provides some evidence that the enzyme has two nucleotide binding sites, each one leading to the formation of a complex emitting mainly at 575 nm (ATP) or

610 nm (dATP). AMP is able to displace dATP from the second site (610 nm) to the first one. Photoaffinity labelling of the second site by 8-azido-AMP gives similar results. The amplification effect of CoA and acetyl-CoA is also reconsidered according to this model.

INTRODUCTION

Firefly luciferase (EC 1.13.12.7) catalyses the oxidation of luciferin in the presence of ATP to produce light with a quantum yield of 0.9, according to the following scheme:

Depending on the relative concentrations of substrates and enzyme, the light is emitted either as a flash or a glow with a slow decay. Despite many studies, the mechanism of the light-emission reaction has not been fully clarified [1–4]. Addition of CoA, CTP, and various ATP analogues can modulate the kinetics of light production [5–9]. Several regulation mechanisms have been proposed that generally involve nucleotide analogues: Ugarova and co-workers showed a regulatory effect of ATP and its analogues on *Luciola mingrelica* luciferase [10–12]. As early as 1969, McElroy et al. [2,13] carried out direct binding measurements on ATP and luciferin. They found that 1 mol of MgATP and luciferin was bound and that 2 mol of ATP can be bound per mol of luciferase. They suggested that the second ATP site may serve a regulatory function. DeLuca and McElroy [3] suggested that the two different rates of light emission they observed were due to two ATP binding sites. Another hypothesis is that luciferase could transfer the AMP group of luciferyl-AMP to ATP to produce *P*¹, *P*¹-di(adenosine) 5'-tetrphosphate [14]. Ford et al. [15] also proposed the existence of at least two binding sites, suggested by the effect of CoA analogues on luciferase activity.

Chemical modifications and mutagenesis studies have failed to identify any amino acid residue involved in substrate binding or enzyme catalysis [16–19]. The crystal structure of *Photinus pyralis* luciferase was published by Conti et al. [20], but without bound substrates or other ligands as noted by Baldwin [21]. Some amino acids (S198, K206, Y340, T343, E344, E389 and Y401) are suspected to be in the active site [22]. Recently it was proposed that a peptide (²⁴⁴HHGF²⁴⁷) obtained through photo-inactivation by a benzophenone-luciferin analogue may locate at or near the luciferin binding site [23].

This study is focused on providing some evidence for the existence of two nucleotide triphosphate binding sites.

MATERIALS AND METHODS

Materials

Luciferase was purified from desiccated firefly tails (Sigma, St. Quentin Fallavier, France), as described by Belinga et al. [24]. A stock solution (1 mg of protein/ml) of luciferase was prepared in 25 mM Hepes (pH 7.75)/4 mM D,L-dithiothreitol/2 mM EDTA/10 mM Mg-acetate/0.1 g/litre BSA. This stock solution was divided into aliquots and stored at –20 °C. D-Luciferin was obtained from Boehringer Mannheim (Meylan, France) and dissolved at 2 mg/ml in an assay buffer: 50 mM Tris, adjusted to pH 7.75 with acetic acid, containing 10 mM Mg-acetate, 4 mM D,L-dithiothreitol, 2 mM EDTA, 0.1 g/litre BSA. The luciferin solution was divided into aliquots and stored at –20 °C. For light-emission assays, the luciferase stock solution was diluted at appropriate concentration with the assay buffer. ATP, 2'-dATP, AMP, acetyl-CoA, and CoA were obtained from Boehringer Mannheim. 8-Azido-AMP, 2'-dAMP, desulpho-CoA and hexokinase attached to beaded agarose were purchased from Sigma. The purity of the nucleotides and their derivatives was checked by capillary electrophoresis.

Preparation of CoA

Commercial CoA contains ATP as the main impurity. This ATP may be responsible for light emission, particularly when examining the effect of CoA at low ATP concentrations. To eliminate this ATP impurity, CoA solution is pretreated with hexokinase as follows: the reaction mixture (4 ml, pH 8) containing 50 mM Tris, 4 mM Mg-acetate, 1 mM glucose, 20 mM CoA and hexokinase attached to beaded agarose (10 units) is incubated for 4 h with agitation at room temperature. Hexokinase is then eliminated by filtration, and the filtrate is stored at –20 °C.

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Table 1 The emitted light energy obtained from the area under each spectrum, normalized versus the value at pH 7.8

pH*	Relative light-emitted energy	
	ATP	dATP
6.8	0.40	0.04
7.3	0.55	0.10
7.8	1.00	1.00
8.3	0.44	0.48
8.8	0.37	0.35

* Ionic strength not corrected.

Measurement of light emission with the luminometer

The effects of nucleoside monophosphates (AMP or dAMP) on the luciferase light emission were determined by using an Optocom I luminometer (MGM Instruments, Handen, CT, U.S.A.). The light emission was integrated for 5 s, without any delay after luciferase injection, at room temperature. The reaction was started by the automatic injection of 50 μ l of luciferase (50 μ g/ml) into a tube containing 50 μ l of assay buffer with 140 μ M luciferin and 5 μ l of nucleotide solution. All the experiments were done in triplicate and the mean values were recorded.

Emission spectra

Emission spectra were recorded on a laboratory-made spectroluminometer with a 3 nm bandwidth and a spectral range of 400–700 nm. The detector was a photomultiplier tube, adjusted for low dark current (Hamamatsu R1462P), with a photocathode cooled by cold water, in order to decrease the noise. To correct for the spectral sensitivity of the photomultiplier tube, the spectroluminometer was calibrated with a secondary standard of spectral irradiance [25].

In the experiments performed to measure the bioluminescence spectra, the reaction mixtures were in a final volume of 1 ml; 950 μ l of the assay buffer containing 130 μ M D-luciferin and luciferase (50 μ g/ml). The reaction was started with the addition of ATP or dATP (50 μ l; respective final concentrations, 5 or 25 μ M) and different additives (AMP, dAMP, CoA, etc.).

pH effect on spectral emission of luciferase

Emission spectra were recorded as described above except that the pH of the Tris-acetate buffer was varied between 6.8 and 8.8 by half-unit steps.

Measurement of light emission with the spectroluminometer

Our spectroluminometer was also used to perform light measurements at a fixed wavelength (575 or 610 nm for ATP or dATP respectively). With a delay of 1 min after mixing, light was measured every second during 10 s and the mean values were recorded. Other conditions were as described for emission spectra.

Photoaffinity labelling with 8-azido-AMP

Photoaffinity labelling was performed at 4 °C with the following reaction mixture: 50 mM Tris-acetate (pH 7.75)/10 mM Mg-acetate/0.1g/litre BSA/1 mg/ml luciferase in the absence or

with 5 $\times$ or 25 $\times$ molar excess of 8-azido-AMP in comparison with the enzyme. The mixture was illuminated for 15 min at 256 nm with a hand-held ultra-violet lamp (3 mW/cm²). After irradiation, excess azido-AMP was eliminated by PD-10 gel filtration (Pharmacia, Orsay, France). The samples were used to record the emission spectra as described above.

RESULTS

pH effect on spectral emission

Whatever the nucleoside triphosphate used, the maximum emission of light occurred at pH 7.8 (Table 1). dATP in the luciferase reaction emitted light with a peak wavelength of 610 nm, and its spectral pattern contained a contribution from the emission centred at 575 nm, corresponding to the maximal emission for ATP (Figure 1).

Inhibition of light emission by nucleoside monophosphates

The effect of AMP and dAMP on the luciferase was measured with a luminometer in the presence of ATP or dATP as substrates. In the presence of ATP, AMP inhibited the luciferase activity as previously described [2] and dAMP showed no inhibitory effect at low concentration (< 25 μ M) but a slight inhibition at higher concentrations (Figure 2A). As compared with the ATP system, the addition of AMP to the dATP system produced a remarkable increase in the light emission. The amplification of light production by dAMP was less effective than by AMP (Figure 2B).

Emission spectra in the presence of nucleoside monophosphates

With ATP as substrate, AMP did not change the spectrum of the light emission ($\lambda_{\text{max}} = 575$ nm) but induced an inhibitory effect of 20 % at 25 μ M (Figure 3A). This inhibitory effect was similar to that observed within the luminometer. The effect of AMP on the dATP emission system increased dramatically the light emission and induced a complete change of the emission spectra (Figure 3B).

dAMP was less effective than AMP at inducing spectral changes in the presence of dATP (results not shown). We think that this is due to the low affinity of dAMP for luciferase.

Figure 1 Normalized bioluminescence spectra obtained at pH 7.8 with ATP (solid line) and 2'-dATP (dashed line)

The relative light energy centred at 575 nm was 0.75 and 0.1 for ATP and dATP respectively.

Figure 2 Effect of AMP and dAMP on the light emission of luciferase

The light peak was recorded with a classical luminometer (peak sensitivity at about 420–440 nm) in the presence of ATP (A) or dATP (B). The concentrations of AMP (■) or dAMP (●) varied between 0 and 100 μM. Each point represents the mean $\pm$ S.E.M. of triplicate measurements.

Effect of CoA or its analogues on light production

The modification of light emission by CoA or its analogues was measured at a fixed wavelength (575 or 610 nm for ATP or dATP respectively) with the spectroluminometer. Table 2 summarizes the results of this amplification. At a low concentration of ATP (0.25 μM), CoA was slightly active and only acetyl-CoA showed a good stimulation of light production. The enhancement by acetyl-CoA was not due to a contamination, as its purity was checked. CoA and acetyl-CoA, at a higher concentration of ATP (0.25 mM), showed a marked enhancement of firefly luciferase activity. In all cases with dATP, CoA and acetyl-CoA were poor enhancers. Desulpho-CoA was also tested; there was no noticeable amplification in any experiments.

Effect of CoA or its analogues on the emission spectra of luciferase

At low and high concentrations of ATP or dATP, neither CoA nor CoA analogues (acetyl-CoA and desulpho-CoA) induced any spectral change, but CoA and acetyl-CoA showed an amplification as described above (results shown only for CoA in Figure 4).

Figure 3 Light-emission spectra with ATP (A) and dATP (B) in the presence of AMP

The concentrations of AMP added to the luminescence reaction were 0 (○), 25 (△) and 100 μM (●) respectively.

To look for a simultaneous effect of AMP and CoA on the spectra, they were added together in the firefly luciferase system. At both high and low concentrations of ATP (Figure 4A), the addition of AMP and CoA showed an inhibitory effect without spectral change. Because the combined effect of CoA and AMP was equivalent to AMP alone, and as CoA in excess could not cancel the inhibitory effect of AMP (results not shown), it seems that this inhibitory effect did not result from competition between CoA and AMP.

With dATP, CoA/AMP addition induced an amplification of the light production and a spectral change (Figure 4B). In this case, CoA could not inhibit the spectral change induced by AMP.

Effect of photo-labelling with 8-azido-AMP

To confirm the existence of a low-affinity nucleotide site, we used photo-insertion of 8-azido-AMP. After irradiation of luciferase with a $5 \times$ or $25 \times$ molar excess of 8-azido-AMP, the emission spectra were recorded in the presence of ATP or dATP. Using ATP as substrate, no spectral change was recorded (result not shown), whereas dATP shifted the light towards shorter wavelengths (Figure 5).

Table 2 Effect of CoA or its analogues on the light emission of firefly luciferase

The amplification is calculated through $(v - v_0)/v_0$, where v_0 is the light production without CoA or its analogues and v is the production in the presence of CoA or its analogues. Values shown are the means $\pm$ S.E.M of triplicate measurements on a spectroluminometer at 575 or 610 nm for ATP and dATP.

Compound	Concentration (μ M)	Concentration ...	Amplification of light emitted			
			ATP		dATP	
			0.25 μ M	0.25 mM	25 μ M	1 mM
CoA	100		0 ± 0.02	2.1 ± 0.07	0.08 ± 0.01	0 ± 0.02
	250		0.23 ± 0.05	2.6 ± 0.06	0.17 ± 0.04	0.017 ± 0.05
Desulpho-CoA	250		0 ± 0.01	0 ± 0.02	0 ± 0.01	0.09 ± 0.03
Acetyl-CoA	100		1.00 ± 0.03	1.33 ± 0.03	0.13 ± 0.03	0.09 ± 0.02
	250		1.29 ± 0.04	1.56 ± 0.05	0.17 ± 0.02	0.13 ± 0.04

Figure 4 Effect of CoA on the light-emission spectra of ATP (A) and dATP (B) in the presence of AMP

The spectra were recorded without additives (○), with 200 μ M CoA (●), with 100 μ M AMP (△) and with 200 μ M CoA/100 μ M AMP (▲).

DISCUSSION

At high concentrations of ATP, the post-flash complex decay of the light production has been explained by two non-exclusive hypotheses: either a rather stable luciferase–oxyluciferin complex that slowly regenerates the free enzyme [5] or the existence

Figure 5 Normalized emission spectra of luciferase in the presence of dATP with the luciferase photo-labelled with 8-azido-AMP

A indicates non-irradiated or irradiated without azido-AMP, B and C indicate irradiated with $5 \times$ or $25 \times$ molar excess of 8-azido-AMP over the concentration of luciferase respectively. Conditions of irradiation: 15 min at 256 nm (3 mW/cm²), 4 °C.

of two ATP binding sites with low and high affinity [3]. This latter model implies that a high-ATP-affinity site is responsible for the slow-decay light emission, whereas a low-affinity site is inhibited after the initial flash and may play a regulatory role of luciferase activity [3].

Our results clearly support the second hypothesis. As the optimum pH of light emission is the same for ATP or dATP (Table 1) in the same buffer, it looks obvious that the luciferase should be in the same conformation in both cases.

Effects of AMP on the ATP system

The inhibitory effect of AMP is not responsible for any spectral change. Thus AMP is thought to compete with ATP at both binding sites: it seems this competition is more potent at the high-affinity site (Figure 3A, inhibition greater at 575 nm than at 610 nm).

Effects of AMP on the dATP system

The addition of AMP in the dATP system induced a spectral shift of the light emission (from 610 to 575 nm) and a large

amplification. If we accept that AMP displaces dATP from the low- to the high-affinity site, two reasons can be stated to explain this amplification. An instrumental reason is that the wavelength of the emission affects the light-detection efficiency of a standard luminometer. Because its peak sensitivity is at about 420–440 nm (S20 photocathode), the shift of the emission towards shorter wavelengths will lead to an increase in the recorded light intensity. A biochemical reason is that, as it seems difficult to assume that the quantum efficiency of dATP is higher than that of ATP, a better proposal might concern the half-life of the enzyme–luciferin–nucleotide complex, shorter with dATP on the high-affinity site.

Effect of 8-azido-AMP

Our results suggest that the affinity of AMP is higher than that of dATP, at least for the low-affinity site. In order to check this hypothesis, we tried to probe the low-affinity site with 8-azido-AMP. This photo-affinity labelling shows a spectral shift towards shorter wavelengths. This change is similar to that obtained with AMP but without amplification. This could be due to the covalent binding on the low-affinity site, leading to a less-efficient enzyme catalysis.

Role of CoA

CoA actually increases the light production in the presence of ATP but not of dATP. Acetyl-CoA, devoid of any reactive free thiol group, amplifies the light production for both, but desulpho-CoA does not (Table 2). These results could be explained by two known models. (i) Trapping of dehydroluciferin by CoA–dehydroluciferin formation from an E–dehydroluciferin–AMP complex [26]. Covalent bond formation between CoA and dehydroluciferin requires the thiol group of CoA to be free. (ii) The liberation of the free enzyme from the E–oxyluciferin complex by conformational changes [5]. According to this model, CoA would participate to the stimulation of the light production only on the low-affinity site [15]. In this model, either the adenine ring or the thiol group of CoA should induce conformational changes to release the enzyme.

Our results are not explained by the two models proposed above. As acetyl-CoA is an enhancer, it may be that the free SH group is not essential for amplification, but the electronegativity of the sulphur atom may play a role that remains to be determined.

Two-nucleotide binding-site model

To explain the two different light emissions (ATP at 575 nm and dATP at 610 nm) in the firefly luciferase system, some hypotheses have been introduced: they involve two ionic states (enolic or keto form) for oxyluciferin [27,28], free-rotation restriction in the C2–C2' bond of oxyluciferin within the active site [29], or the environmental polarity of the oxyluciferin binding site [30]. They are based on the state of ionizable groups affected by pH changes; that is, oxyluciferin or amino acids in the active site. Conformational changes induced by AMP or dAMP binding can modify the environment of the active site and the binding state of oxyluciferin. Nevertheless it is difficult to assume that only these conformational changes can modify the dAMP–oxyluciferin–E complex to emit mainly at shorter wavelengths (575 nm).

In comparison, our data, based on a spectral change induced by competition with AMP, are easily explained if we accept the existence of emission at two wavelengths attributed to two nucleotide binding sites. It is assumed that the emitting species is a luciferyl–enzyme complex and, for example, it is accepted that the mutation of an amino acid not directly involved in the catalytic site influences the emission wavelength [31]. Our results provide evidence for the existence of two nucleotide sites and strongly suggest that the conformation of the emitting species is dependent on the nucleotide used to obtain it, through a conformational change induced by nucleotide monophosphate. The question remains as to whether this conformational change is exerted directly on the emitting species or through the protein.

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