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Firefly Luciferase Generates Two Low-Molecular-Weight Light-Emitting Species

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A bioluminescent D-luciferin-luciferase mixture is separated by gel filtration during the time course of the reaction. A simultaneous analysis with an UV-visible diode array detector and an on-line luminometer gives nonsuperimposable chromatograms. Luminescence recordings display three peaks, one associated with the enzyme (light-emitting species 1: LES_1), and two other species free from the luciferase: LES_2, with a luciferyl-adenylate-like spectrum and LES_3. Production of these two species is nucleotide (ATP or 2′-dATP)- and pH-dependent. The chromatographic data presented here could lead to reconsideration of the generally assumed emission mechanism, which involves one emitter only. It could also suggest that each free emitting species is related to a colour of emission corresponding to the two defined wavelengths previously described (~575 and ~620 nm).

Luciferase from the North American *Photinus pyralis* fireflies (EC 1.13.12.7) is now widely known because of its numerous applications in industry and medicine for ATP detection (1, 2). Cloning its gene (3) has opened up new opportunities in cell biology, using luciferase as a reporter gene (4–8). Moreover, it allowed an accurate knowledge of the enzyme by sequence homology and 3D structure studies (9), and the incidence of mutations on catalytic activity and light emission (10). Luciferase (E) catalyses the oxidation of D-luciferin (LH_2) in the presence of ATPMg to generate light, according to the simplified following scheme, generally proposed:

$$E + LH_2 + ATPMg \rightleftarrows E - LH_2 - AMP + O_2 \rightarrow$$
$$OxL + light + AMP + CO_2 + PPi$$

Abbreviations used: 2′-dATP, 2′-deoxyadenosine 5′-triphosphate; LES, light-emitting species; PLES, product derived from light-emitting species.

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If the enzymatic reaction is initiated by adding excess of substrates to luciferase, a flash of light followed by a rapid decay towards a low level of light emission is obtained. This decay was initially attributed to a product inhibition of the enzyme (OxL: oxyluciferin) (11), leading to a slow turnover of luciferase. It was also suggested (12, 13) that the two different rates of light emission were due to the existence of two ATP binding sites: a low affinity one, inactivated after the initial flash, and a higher affinity site catalysing the continuous low production of light. This model was supported by kinetic studies (14, 15) showing that other nucleotides or coenzyme A could only enhance light emission at high ATP concentrations. The same authors (16) reconsidered the effects of AMP and dATP upon light emission, already described (13), and made the assumption that a single ATP binding site, able to take different conformations, could also explain the observed kinetics. Based on the same assumptions, a luciferase activity regulatory function of ATP has been described (17), leading the authors to propose the presence of an allosteric binding site for ATP. On the other hand, it was recently shown by spectroluminescence measurements (18), that the dual wavelength emission of the bioluminescent reaction (575 and 610 nm) could be attributed to two nucleotide binding sites, each one leading to the formation of a complex emitting mainly at 575 nm with ATP or 610 nm with 2′-dATP. As suggested (11), this work is an attempt to go further in the understanding of biochemical mechanisms, which underlie light emission.

MATERIALS AND METHODS

Materials. Firefly luciferase (*Photinus pyralis*) was purchased from Roche Molecular Biochemicals (ref: 634 409, Meylan, France). A stock solution of luciferase was prepared by dissolving 1 mg in 1 ml of 50 mM Tris-Acetate buffer pH 7.5 and stored at −20°C. D-Luciferin, 2′deoxy-ATP and ATP were purchased from Roche Molecular Biochemicals and prepared as stock solutions every day at 10 mM. The reagent buffer used in all experiments consisted in 25 mM Tris, 10 mM Magnesium Acetate, 2 mM EDTA adjusted to pH 7.75.

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Methods. The reaction was triggered by adding ATP or 2′deoxy-ATP (100 μM both) to luciferase 0.1 mg/ml and D-luciferin 100 μM in the reagent buffer. Luciferase and D-luciferin concentrations remained constant in all experiments. Bioluminescent mechanisms of the firefly luciferase reaction were sought for by gel filtration through a 1.1 × 8 cm column of Trisacryl GF05M (IBF biotechnics, Villeneuve-la-Garenne, France) equilibrated and eluted at a constant flow rate (0.85 ml/min) with the reagent buffer. Trisacryl GF05M has an exclusion limit of 3 kDa and a linear fractionation range from 200 to 2500 Da. In these conditions, luciferase (62 kDa) is excluded from the gel at the void volume, and ATP and D-luciferin are separated according to their molecular weights. The column is fit into a ThermoQuest HPLC integrated system including a P4000 pump, an autosampler (AS3000) and an highly sensitive UV-visible diode array detector (UV6000). It is followed by a home-made on-line luminometer with a 200 μl quartz circulation cuvette placed in front of a side-on multialkali photocathode photomultiplier (PM) tube (Hamamatsu R928) with a spectral detection range from 220 nm to 800 nm. Its anodic current is converted into voltage and amplified through an operational amplifier specially designed for very low input currents. In order to decrease thermal noise the luminometer is cooled to 5°C. The output signal is recorded and treated simultaneously with absorbance chromatograms.

Luciferase catalysed reaction tracking. The bioluminescent reaction medium containing ATP or 2′deoxy-ATP, together with luciferase and D-luciferin is incubated on the AS3000 at 25°C and 50 μl of the reaction mixture is injected every 20 min from t_0 (1 min 30 s) to t_0 + 120 min.

Protein assay. The bicinchoninic acid protein assay from Pierce (Rockford, IL) (enhanced protocol: sample tubes are incubated at 60°C for 30 min) is performed on 0.5 ml fractions collected from the chromatographic elution of a complete reaction mixture containing 0.2 mg/ml luciferase, ATP and D-luciferin (100 μM both). The protein assay is standardized with 0.5 ml luciferase dilutions ranging from 2 to 20 μg/ml.

Chromatographic controls. Two successive independent chromatographic elutions are carried out: first, a mixture of ATP and D-luciferin (100 μM both) is submitted to chromatography, and 0.5 ml fractions are collected. Then a second chromatographic elution is made with luciferase alone (0.1 mg/ml) in the same conditions and the fractions are collected in the corresponding vials used for the previous run.

The same procedure is applied to the enzyme in the presence of at least one of the two substrates: a first chromatographic elution is made with ATP or D-luciferin alone, and the next one respectively either with luciferase and D-luciferin, or luciferase and ATP. Luminescence of each reassociated fraction is assessed with an Optocomp I luminometer (MGM Instruments, Hamden, CT).

pH effects. Incidence of pH modifications upon light emission of the reaction was assessed as described above and also at pH 5.5 and 6.5. The pH of reagent and elution buffers was modified consequently.

Mathematical processing of chromatograms and spectra. The chromatograms are treated through a specially designed algorithm (19), in order to extract hidden peaks from chromatographic data. Though working on any bell shape, the assumption is made in this study that we are dealing with Gaussian peaks. The first step is to adjust a Gaussian curve on the main peak, through a single exponential regression on the part of the chromatogram, which is supposed not to be contaminated by the second peak. From this fit, a reference single peak chromatogram is determined. A comparison with the experimental trace is carried out through Rix algorithm after normalization of the two chromatograms. The intersection of the latter gives the position of the second maximum, and the relative height of the second peak comes out from a linear regression between original and shifted chromatogram values.

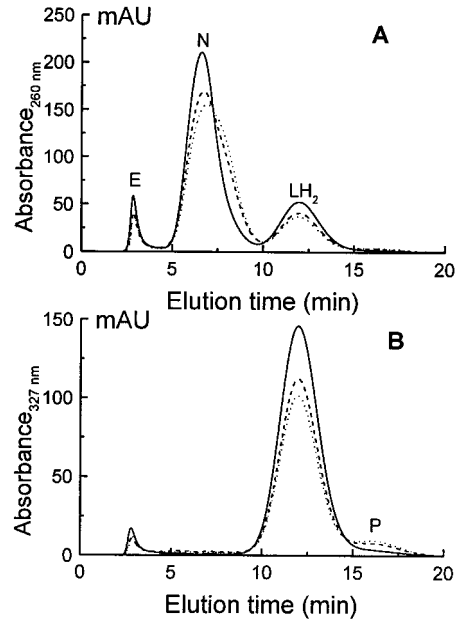

FIG. 1. A typical chromatographic separation of reactants by gel filtration, recorded at 260 nm (A) and 327 nm (B). These chromatograms are representative of three different experiments, as bioluminescent reaction goes on: after (—) 1 min 30 s, (–) 60 min and (· · · ·) 120 min of incubation.

Spectral shape comparisons are carried out through shape factor computation (20).

RESULTS AND DISCUSSION

Chromatographic Separation of Reactants

On Fig. 1A, the chromatogram acquired at 260 nm shows a typical separation obtained at three different reaction times (t_0, t_0 + 60, t_0 + 120 min) of the same bioluminescent mixture. Control injections with luciferase, or ATP, or D-luciferin alone let us confirm the retention times obtained in Fig. 1. The first peak is luciferase (E) with a retention time ($Rt_{Luciferase}$) of 2.9 min confirmed by a maximal absorption at 280 nm (data not shown). The second (N) and the third (LH_2) ones are respectively ATP (Rt_{ATP} = 6.6 min) and D-luciferin (Rt_{LH2} = 12 min), which are confirmed by their spectra. Decrease of ATP observed on Fig. 1A is concomitant with the appearance of a shoulder that could be due to an increase of AMP, its reaction product. This was confirmed either by a Gaussian decomposition of the nucleotide peak or by injection of AMP alone: AMP is eluted after ATP with a retention lag time corresponding to the difference observed between the peak (Rt_{ATP} = 6.6 min) and the shoulder (Rt_{AMP} = 7.9 min). On Fig. 1B recorded at 327 nm, λ_{max} of D-luciferin at pH 7, it is noteworthy that, as the reaction goes on, D-luciferin is consumed and an absorption band at 327 nm appears, associated with the enzyme peak. A fourth product (P), detected at Rt = 15.7 min,

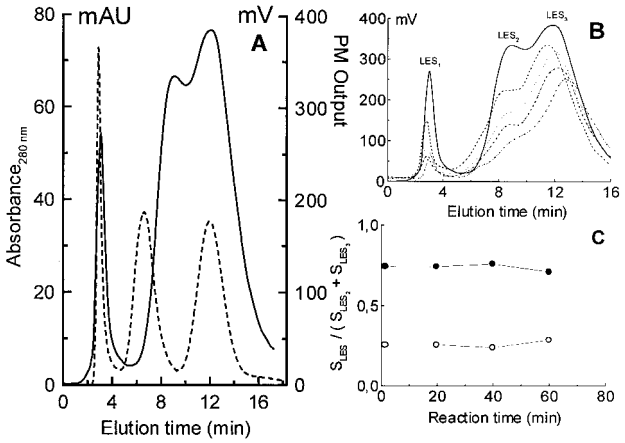

FIG. 2. Superimposition of UV and luminescent recordings (A) and evolution of luminescent chromatograms during the time course of the reaction (B). (A) Chromatograms of the reaction mixture obtained after 1 min 30 s of incubation: (–) UV trace at 280 nm (left axis scale) and (—) luminescent recording (mV axis scale on the right). (B) The reaction was followed up during the time: luminescent chromatograms are shown after (—) 1 min 30 s, (–) 20 min, (····) 40 min, (-·-·-) 60 min and (-··-··-) 80 min of incubation. (C) Relative surfaces (●) under the third luminescent peak (LES_3) and (○) under the second luminescent peak (LES_2) versus the total surface under LES_2 and LES_3. Each point is the mean of three different measurements.

absorbs more at 327 than 260 nm, indicating that it could be also a D-luciferin derivative accumulating from 1 min 30 s to 120 min as a final product of the reaction, spectrally analysed later.

Luminescence Detection

The home-made luminometer is connected to the UV-visible diode array in order to detect eventual luminescent products. On Fig. 2A, the absorbance recording at 280 nm (dashed line) and the bioluminescent chromatograms of the reaction mixture incubated 1 min 30 s are superimposed. The luminescent chromatogram (solid line) exhibits three peaks: the first emitting peak (light-emitting species 1: LES_1) with a retention time similar to the enzyme which corresponds to a D-luciferin derivative associated with the luciferase peak. The second one (LES_2) (Rt_{LES2} = 9.3 min) is eluted between ATP and D-luciferin absorption peaks. It is possible to extract at the corresponding elution time its absorption spectrum between 240 and 500 nm (Fig. 3B). The third luminescent peak (LES_3, Rt_{LES3} = 13 min), closely eluted after LES_2, strictly overlaps D-luciferin peak decay. Figure 2B displays the evolution of the luminescent chromatograms during the time course of the reaction. A continuous decrease of light intensity of each species is observed (detector output from 280 to 50 mV for LES_1, 350 to 100 mV for LES_2 and 400 to 250 mV for LES_3). Contributions of LES_2 and LES_3 were analysed by a Gaussian decomposition of these two peaks as described above. The surfaces under each separated peak were calculated and related to the sum (LES_2 + LES_3). Results are plotted on Fig. 2C for three experiments in the same conditions between 0 and 60 min, after the beginning of the reaction. The surface ratios remain constant during time, indicating that the rate of production of the two free light-emitting species remains in the same proportions during the reaction.

Control Experiments

In order to check if the presence of the two low molecular weight light-emitting species (LES_2 and LES_3) is not due to a potential contamination by small amounts of the enzyme creeping along the column, two complementary experiments are carried out: chromatographic controls, and a protein assay (see Materials and Methods section).

The light emitted from the reassociated fractions of the three chromatographic controls is compared with that obtained with 0.5 ml fractions from a complete reaction mixture. No significant luminescence is observed in any control vial, whereas fractions collected from the complete reaction sample give a luminescent chromatographic pattern similar to Fig. 2A. The protein measurements made on three successive runs showed that more than 95% of the enzyme loaded (10 μg per run) onto the column was eluted in the fractions collected at Rt = 2.5 min and Rt = 3 min, times surrounding the luciferase peak ($Rt_{Luciferase}$ = 2.9 min) shown on Fig. 1. No detectable protein amount was found in the other fractions (i.e., less than 0.5 μg per tube). It seems that the results of the chromatographic controls and protein measurements allow us to

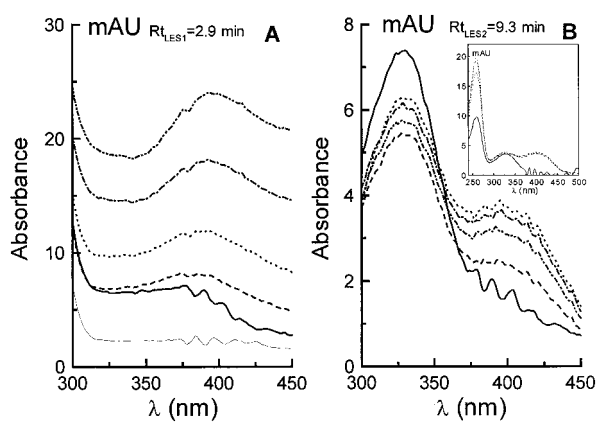

FIG. 3. Spectral characterization of LES_1 (A) and LES_2 (B) during time course of the reaction. Data extracted at (— bold line) 1 min 30 s, (–) 40 min, (····) 60 min, (-·-) 80 min and (-···-) 100 min after the beginning of the reaction. At (A) Rt = 2.9 min (luciferase peak) between 300 to 450 nm; (B) at Rt_{LES2} = 9.3 min, from 240 to 500 nm (inset) and 300 to 450 nm. (A) (— thin line) Luciferase injected alone as a control.

exclude that LES_2 and LES_3 are chromatographic artefacts.

Spectral Analysis of Light-Emitting Species

For a better characterisation of light-emitting species, absorption spectra corresponding to retention times of LES_1 and LES_2 are extracted from UV chromatograms. The detection range of the diode array was first set between 240 and 500 nm, but significant modifications of spectra are noted between 300 and 500 nm for LES_1 (Fig. 3A) and LES_2 (Fig. 3B), as the reaction goes on. In a second set of experiments, the detection range was reduced between 300 and 450 nm to obtain accurate spectral data for these compounds. Even in these conditions, the co-elution of LES_3 with D-luciferin prevents its spectral analysis.

Figure 3A displays clearly a continuous increase of the absorption at 395 nm from 1 min 30 s to 100 min, pointing out a product accumulation within the enzyme peak as the reaction goes on. Concurrently, the light emission of LES_1 (Fig. 2B) decreases continuously. Since the light emission is related to the reaction rate, it cannot be directly compared to absorbance values without being integrated over the reaction time. A comparison of the absorbance increase at 395 nm (Fig. 3A) with the cumulated light intensity of LES_1, from 1 min 30 s to 80 min is shown on Fig. 4B. These data suggest that the absorbance recordings at 395 nm (Fig. 3A) may reflect the concentration increase of a LES_1 radiative desexcitation product bound to the enzyme ($PLES_1$). According to the isolation of the luciferase-product complex performed by Gates *et al.* (11), and previous spectral data (21, 22), $PLES_1$ should be oxyluciferin (base form pH >9, λ_{max} = 393 nm).

The evolution of absorption spectra of LES_2 between 240–500 nm (inset of Fig. 3B) exhibits two main features: an absorption increase at 260 nm corresponding to a nucleotide moiety and the appearance of an absorption band at 395 nm corresponding to a D-luciferin derivative. We recall that ATP, ADP and AMP elute between 6.6 min and 7.9 min, completely out of the range from this species (Rt = 9.3 min). Nevertheless, LES_2 obviously contains a nucleotidic moiety, and the spectra shapes suggest that LES_2 is a D-luciferin-adenylate complex (23, 24). Assuming that LES_2 is a stoichiometric association of AMP and D-luciferin, we compared its chromatographic absorbance ratio A_{260}/A_{280}, measured at Rt = 9.3 min, with the ratio of the molar absorption coefficients of pure AMP and D-luciferin at 260 and 280 nm $((\varepsilon_{AMP_{260}} + \varepsilon_{LH2_{260}})/(\varepsilon_{AMP_{280}} + \varepsilon_{LH2_{280}}))$. The latter was obtained in a spectrophotometer cuvette, in the reaction buffer at pH 7.75. The same value of 3.8 ± 0.2 was obtained for the two ratios, in good agreement with the assumption described above. It may be surprising to find the luciferyl-adenylate eluted after nucleotides, though its molecular weight is higher. Nevertheless, the interaction between filtration beads and molecules is rather complex, and involves both steric and artifactual ionic interactions. The odd position of LES_2 cannot be taken into account to discard the luciferyl-adenylate nature of the compound, attested from spectral results.

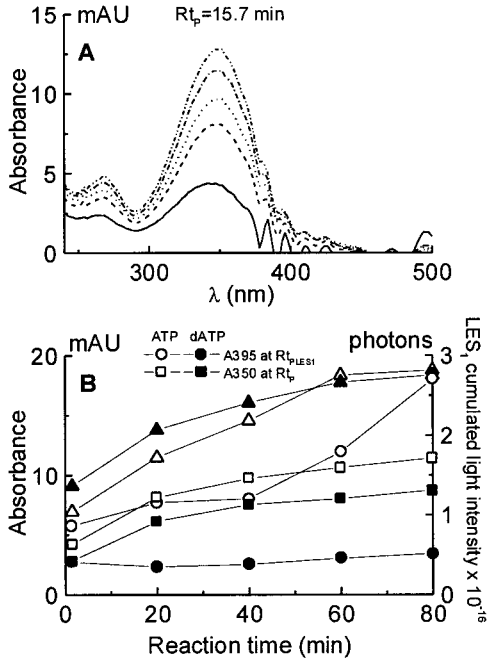

FIG. 4. Spectra of the product P during the time course of the reaction (A). Increase of its absorbance and that of $PLES_1$ compared with LES_1 cumulated light intensity (B). (A) The spectra of P are extracted at Rt = 15.7 min during the time with ATP as substrate, at (—) 1 min 30 s, (–) 20 min, (· · ·) 40 min, (- · -) 80 min and (- · · -) 120 min after the beginning of the reaction. (B) Absorbances (left axis scale) (i) at 350 nm of the final product P (Rt_P = 15.7 min) (squares) and (ii) at 395 nm under the luciferase peak (Rt_{PLES1} = 2.9 min) (circles) compared with LES_1 cumulated light intensity (triangles, right axis scale), during time course of the reaction, with (○, △, □ ATP or (●, ▲, ■) 2′-dATP.

Spectra recorded from 300 to 450 nm (Fig. 3B) looks like D-luciferin spectra submitted to a change of pH from acidic to alkaline (22). The absorption increase at 395 nm occurs together with a decay at 330 nm, suggesting a long-wavelength shift (330 → 395 nm), due to pK_a variations (the pH of the elution buffer is maintained at 7.75). In order to confirm this hypothesis, a computerized shape comparison between each LES_2 spectrum and D-luciferin spectra acquired in the elution buffer with a pH varying from pH 7 to pH 9 (every 0.2 pH units) was carried out. Table 1 shows the best shape comparison and the standard deviation (SD) obtained for. As expected, the wavelength shift observed (Fig. 3B) between 1 min 30 s and 60 min is equivalent to a pH change of the D-luciferin from 7.8 to 8.6. Since the elution buffer cannot change its pH during the elution process, the only explanation is a change on the pK_a of the ionized D-luciferin moiety of the LES_2 mol-

TABLE 1

Shape Comparison between Spectra of LES_2 during Time and pH-Dependent Spectra of D-Luciferin

Spectra of LES_2: Reaction time (min)	Spectra of D-luciferin: Closest pH	SD
1.5	7.8	0.09
20	8.2	0.17
40	8.6	0.24
60	8.6	0.25
80	8.4	0.19
100	8.2	0.14
120	8	0.13

Note. The wavelength shift of LES_2 observed between 1 min 30 s and 60 min seems equivalent to that obtained through a pH change of the D-luciferin from 7.8 to 8.6.

ecule, due to modifications of the electronic delocalisation on the phenol ring.

Spectrum of the Product P (Rt = 15.7 min)

The spectra of the last product detected at 15.7 min are displayed on Fig. 4A for ATP as substrate. With 2′-dATP, spectra extracted at the same retention time are identical (data not shown) but the yield of production is reduced (Fig. 4B). In both cases, this product accumulates during the reaction and its maximal absorption wavelength is 350 nm (Fig. 4A).

From early substrate and product absorption studies (21, 26, 27), this compound might be oxyluciferin free from luciferase (acid form pH <9, λ_{max} = 348 nm). Recent results (28), and synthesis of intermediates (24), both showing spectral data about dehydroluciferin (λ_{max} = 350 nm) and dehydroluciferyl-adenylate could suggest that P is one of these two molecules. Nevertheless, dehydroluciferyl-adenylate (L-AMP) was only described as an intermediary leading to the formation of a stable inhibitory luciferase-L-AMP complex. Moreover, from spectra displayed on Fig. 4A, no significant absorption at 260 nm is observed to indicate the presence of an AMP moiety, suggesting that P is not a dehydroluciferyl-adenylate. As dehydroluciferin, a contaminant of D-luciferin, was not described to be synthesized and accumulated during the reaction, the hypothesis that P could be dehydroluciferin, should also be discarded. Therefore, the free final product P and $PLES_1$ seem to be the oxyluciferin detected in two different environments: the observed maximal absorption wavelength (λ_{max} = 395 nm) of $PLES_1$ bound to the enzyme, differs from the maximum of P (λ_{max} = 350 nm) free in the elution buffer at pH 7.75. This could be due to polar interactions of $PLES_1$ on the protein.

Influence of 2′-dATP and pH

The incidence of a pH decrease of the reaction and elution buffers with ATP, and the substitution of ATP by 2′-dATP in the reaction, are shown on Fig. 5 upon luminescence chromatograms.

With ATP as substrate, maximal LES_3 amount is obtained at pH 7.75 and, when pH changes from pH 7.75 to 6.5 or 5.5 (Fig. 5A), total light intensity of LES_3 decreases of 55% and 95%, respectively. LES_2 luminescence does not display significant modifications between pH 7.75 and 6.5 but is quite reduced at pH 5.5. With 2′-dATP instead of ATP (Fig. 5B), at pH 7.75, LES_3 is totally abolished, and the light emission of LES_2 seems to be intensified. Previous authors (21, 25) described a spectral shift of the light emission from yellow-green to red as well as a decrease of the emission yield, respectively with 2′-dATP or a pH decrease. As seen on our chromatograms in Fig. 5A and 5B, these two factors (pH decrease or 2′-dATP) prevent LES_3 synthesis. Our results could logically suggest that LES_2 is related to red light emission and LES_3 to the yellow-green one (in comparison with commercial luminometer, the on-line luminometer has a photocathode with a wide spectral sensitivity, 200–800 nm). Unfortunately, their weak light intensities prevented any spectroluminescence analysis.

The influence of 2′deoxy-ATP was also compared with ATP in terms of product accumulation within the enzyme ($PLES_1$, previously described as oxyluciferin) on Fig. 4B. From t_0 to t_0 + 80 min, the amount of $PLES_1$ in the luciferase peak is two to six times greater with ATP than with 2′-dATP (6 to 18 mAbs versus 3 mAbs). Nevertheless, LES_1 cumulated light intensities, plotted on Fig. 4B versus the time course of the reaction do not depend significantly on the nucleotide used.

Using 2′-dATP instead of ATP displays two major interdependent effects: from a luminescence point of

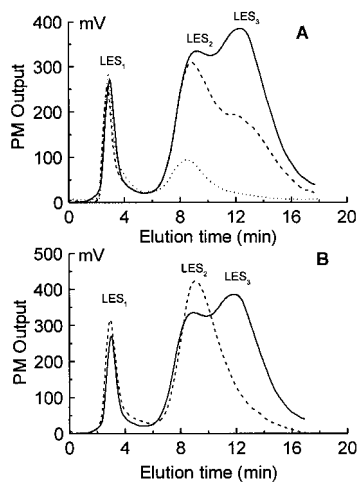

FIG. 5. Nucleotides and pH dependence on the production of the free light-emitting species. Luminescent chromatograms obtained: (A) with ATP when decreasing pH of the reaction and elution buffer from 7.75 (—) to 6.5 (–) and 5.5 (···). (B) With 2′-dATP (–) (100 μM) instead of ATP (—) at pH 7.75.

view, it abolishes LES_3 synthesis, without affecting LES_1 and LES_2 light emissions (Fig. 5), while the end-product formation of $PLES_1$ and P are decreased (Fig. 4B).

Our first goal was to point out the relationship between the emitting enzyme-product complex and the rapid light emission decay during the bioluminescent firefly reaction (11). Luminescence measurements, combined with UV detection (Fig. 2A) confirm the existence of two forms of the complexed enzyme: an emitting LES_1-luciferase and a $PLES_1$-luciferase, which accumulates over the reaction, with absorption maximum at 395 nm. Furthermore, depending on the nucleotide nature and/or the pH of the medium, luciferase also generates one or two free emitting species:

—LES_2, is assumed to be a luciferyl-adenylate obtained mainly with 2′-dATP at pH 7.75 and with ATP at pH 5.5. It might be related to the red light emission (620 nm) of the bioluminescent reaction.

—LES_3, mainly observed with ATP at optimum pH. Its nature was not spectrally elucidated, and it might be related to the yellow-green light emission (575 nm).

These results are in good agreement with our previous work (29) which pointed out the existence of free emitting species by spectroluminescence measurements on a reaction medium in which the triggering immobilized His-tagged luciferase is removed after 1 min. Spectra of the free species (without luciferase) are very similar to those obtained with a complete reaction medium.

On the other hand, we gave strong evidence (18) for the existence of two nucleotide binding sites which are catalytically active: this was based on photoaffinity-labelling experiments of luciferase by 8-azido-AMP in which 2′-dATP was displaced from the second site (620 nm) to the first one as demonstrated by the induced change of the emission wavelength after photolabelling. Therefore, we could hypothesise that the two low molecular weight emitting species characterized by gel filtration (not bound to the enzyme) are generated by two different catalytic active sites.

As suggested by previous authors (16, 17), (i) luciferase may have only one allosteric binding site for nucleotides and (ii) dATP, among other nucleotides, was described to enhance the turnover of the enzyme (16). In this case, LES_2 and LES_3 could not be simultaneously generated by the enzyme and LES_3 should be an excitation product derived from LES_2. Nevertheless, our first hypothesis seems well supported by the comparison of the accumulation rates of the bound ($PLES_1$) and free product (P), obtained with ATP or 2′-dATP, with the cumulated light intensity of LES_1, displayed on Fig. 4B. While $PLES_1$ accumulates much more with ATP than 2′-dATP, the accumulation rate of P is unchanged whatever the nucleotide considered. These results could corroborate the existence of two emitting sites with different light emitting rates (13), provided that the site responsible for the flash emission could be the same which accumulates $PLES_1$, whereas the second one, observed with 2′-dATP in our experiments, could generate a glow light.

Despite many physicochemical and biochemical arguments in favour of the two emitting sites hypothesis, the energy transfer single site model, assuming one emitting species with two different energy levels, cannot be discarded at the moment. A higher sensitivity spectroluminometer has to be built in order to spectrally characterize the emissions of LES_2 and LES_3, and therefore to assign a colour to these two emitting species.

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