Firefly luciferase can use L-luciferin to produce light

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L-Luciferin is a competitive inhibitor of firefly luciferase with a K_i between 3 and 4 μM. Furthermore L-luciferin can serve as an alternative substrate for light production. Catalysis of L-luciferin can be observed in the absence of, or at low concentrations of, D-luciferin. The light production from L-luciferin increases slowly (maximal half-time 8 min) to a stable plateau. At low concentrations of enzyme and L-luciferin, maximal light production is about half of that observed at corresponding D-luciferin concentrations. Increasing the concentration of enzyme or L-luciferin reduces the light production relative to that obtained by D-luciferin catalysis. In contrast to the catalysis of D-luciferin the light production from L-luciferin can be effectively stimulated by the addition of PP_i provided that luciferase is premixed with inorganic pyrophosphatase (PP_i-ase). A flash is emitted if PP_i is injected into a mixture of luciferase, L-luciferin, ATP and PP_i-ase. The system maintains its responsiveness and emits further flashes of about equal duration and intensity upon repeated additions of PP_i. It is proposed that PP_i induces a racemization of enzyme-bound L-luciferyl adenylate. The potential usefulness of PP_i-dependent intracellular ATP monitoring is discussed. The proposed activation of firefly luciferase by PP_i may be part of the regulation of *in vivo* flashing.

INTRODUCTION

Fireflies emit flashes of species-specific duration and frequency as signals for mating and hunting [1]. Advertising males repeat their flash pattern until they receive an answer. This flash dialogue proceeds for five to ten exchanges until the male reaches the female. Females of several *Photuris* species are able to attract males of different species by adjusting their responses to the male flashing pattern. By mimicking their mating signal the females are able to capture and devour the males. There is no doubt that this complex signal repertoire among fireflies is under nervous control [1,2] but the precise biochemical mechanism of deliberate light production is still unknown.

Firefly luciferase catalyses the oxidative decarboxylation of D-luciferin (Figure 1) in the presence of ATP and thereby light is emitted. With a detection limit in the femtomole range light production of firefly luciferase is one of the most sensitive analytical tools for the detection of ATP. Numerous techniques have been developed for biochemical and clinical applications [3,4]. Thus ATP monitoring was successfully used to measure ATP production by intact mitochondria isolated from muscle biopsies in the milligram range [5]. Recently, the potential of the bioluminometric technique was demonstrated by monitoring ATP production from mitochondria isolated from about 1 μg of tissue, i.e. one islet of Langerhans [6].

Attempts to monitor cytoplasmic ATP changes in single isolated cells clearly demonstrated that a deeper understanding of the light production by firefly luciferase is necessary [7]. A major problem in cytoplasmic ATP monitoring experiments is the irreversible loss of enzyme activity at high concentrations of

D-luciferin [8]. It is known from *in vitro* studies that in the presence of high concentrations of substrates firefly luciferase is strongly inhibited after an initial flash due to accumulation of inhibitory oxyluciferin [9]. It was also observed that the injection of high concentrations of luciferase into single cells, which is necessary to obtain a measurable signal, also favours product inhibition during monitoring [8]. So far no conclusive explanation has been proposed regarding how product inhibition after flashing is avoided hence maintaining the responsiveness of the system.

Based on the novel observation that firefly luciferase can emit light in the absence of D-luciferin but in the presence of the catalytically inactive L-luciferin isomer a model is proposed which allows repeated light flashes in response to additions of PP_i.

MATERIALS AND METHODS

Chemicals

Firefly luciferase (EC 1.13.12.7), inorganic pyrophosphatase (EC 3.6.1.1), ATP and electrophoretically homogeneous, freeze-dried BSA were purchased from Boehringer G.m.b.H. (Mannheim, Germany). D-Luciferin (free acid) was obtained from Biothema AB (Dalarö, Sweden). L-Luciferin (free acid of 99 % purity, 0.5 % D-luciferin contamination) was kindly provided by Dr. A. Lundin. Hepes was from Calbiochem (La Jolla, CA, U.S.A.). KOH (Suprapur) and tetrasodium pyrophosphate were from Merck (Darmstadt, Germany). KCl and $MgCl_2$ were of pro analysi grade. Quartz bidistilled water was used throughout. The reagent buffer consisted of 50 mM Hepes containing 20 mM KCl, 5 mM $MgCl_2$ and albumin (0.01 %, w/v) and was adjusted to pH 7.6 with KOH.

Analytical procedure

A modified Aminco microfluorimeter (American Instruments Co., Silver Spring, MD, U.S.A.) was used to detect light emission [10]. The reaction was started by manual injection of 100 μl of
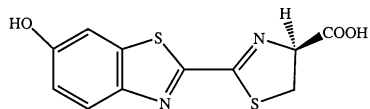

Figure 1   Structure of D-(−)-luciferin

buffer containing enzyme, premixed with ATP, into a micro-test-tube (Milian Instruments S.A., Geneva, Switzerland) mounted in front of the photomultiplier. PP_i or inorganic pyrophosphatase (PP_i-ase) were added to the enzyme solution as indicated. The test-tube contained 7 μl of D- or L-luciferin. All solutions were temperature equilibrated. The test-tube holder was kept at 25 °C. The signal from the fluorimeter was transferred to a Macintosh II fx (Apple Computer Inc., Cupertino, CA, U.S.A.) via an analogue-to-digital converter (ACM2, Strawberry Tree, Sunnyvale, CA, U.S.A.) and analysed with the Workbench program from the same supplier.

Determination of kinetic parameters

Hyperbolic concentration rate dependencies were analysed as described in [11] for a random bireactant mechanism. Hanes plots were constructed and normalized by dividing all values with the highest rate measured for the lowest substrate concentration of the fixed substrate (i.e. 1 μM D-luciferin and 5 μM ATP). This compensates for experimental variations in the enzyme activity and allows a direct comparison of V_{app} values obtained in different experiments. The resulting parameter K_{app} and V_{app} were used in secondary plots to determine K_m, K_S and V_{max}. All mathematical calculations were carried out with the Sigma plot 4.1 program (Jandel Scientific G.m.b.H., Erkrath, Germany).

RESULTS

Inhibition of the peak light production by L-luciferin

The inhibition pattern of L-luciferin at saturating concentrations of ATP was analysed as described by Cornish-Bowden [11]. Dixon and Cornish-Bowden plots were constructed from initial rates obtained in the presence of increasing concentrations of inhibitor. Intersecting lines in the Dixon plot (Figure 2a) together with parallel lines in the corresponding Cornish-Bowden plot (Figure 2b) indicate a competitive inhibition by L-luciferin with a K_i between 3 and 4 μM. Due to the strong binding, the presence of small amounts of L-luciferin significantly affects most kinetic constants determined from initial rates (Table 1). Addition of L-luciferin reduces $K_S^{D\text{-luciferin}}$ and $K_m^{D\text{-luciferin}}$ in a concentration-dependent manner and increases K_m^{ATP}.

The K_S^{ATP} remains unchanged. Maximal activity of firefly luciferase determined from secondary plots for different fixed concentrations of ATP is not significantly inhibited in the presence of 1 % or 5 % L-luciferin. However, maximal activity determined from secondary plots of different fixed concentrations of D-luciferin is reduced to 50 % or 41 % of the uninhibited maximal activity in the presence of 1 % or 5 % L-luciferin respectively. The presence of 10 % L-luciferin further reduces the maximal activity to 34 % (results not shown).

Effect of L-luciferin on steady-state light production

Apart from the inhibition at the time of peak light production L-luciferin modulates the steady-state light production. The stable light production in the absence of the inhibitor changes to a transient inhibition after an initial flash (Figure 3a). L-Luciferin inhibits the post-flash light production in a concentration-dependent manner. The time of reactivation increases with increasing L-luciferin concentrations from 3.4 to 5.8 min at 0.2 and 1 μM L-luciferin respectively. This effect could only be observed at low concentrations of D-luciferin, whereas at high D-luciferin concentrations (e.g. 200 μM) addition of L-luciferin does not induce a transient.

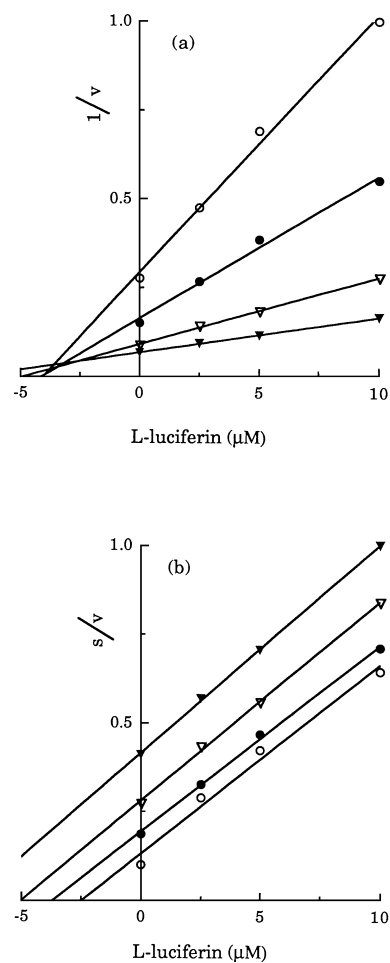**Figure 2 Competitive inhibition of firefly luciferase by L-luciferin**

Dixon plots (**a**) and the corresponding Cornish-Bowden plots (**b**) were constructed from data of initial rates in the presence of various L-luciferin concentrations. Enzyme (5 nM) was premixed with ATP (500 μM). D-Luciferin was present at 10, 20, 50 or 100 μM. Activities in (**a**) were normalized by dividing all values by the lowest observed rate, i.e. at 10 μM D-luciferin in the presence of 10 μM L-luciferin. Activities in (**b**) were normalized by dividing all values by the rate observed at 100 μM D-luciferin in the presence of 10 μM of inhibitor. Solid lines were calculated by linear regression. Mean values of two experiments are given.

At lower ATP concentrations and otherwise unchanged reaction conditions the degree of reactivation exceeds the initial activity leading to a stable amplification of the signal (Figure 3b). Even when amplification predominates a transitional inhibition precedes the reactivation step. L-Luciferin amplifies the light production in a concentration-dependent manner.

Effect of PP_i

PP_i is the first reaction product liberated in the initial adenylation of both luciferin isomers [12,13]. PP_i in the micromolar range is known to stabilize the steady-state activity of firefly luciferase by reacting with enzyme-bound AMP [8,14]. This reduces the forward adenylation rate constant and, in addition, facilitates the product release from the enzyme. The effect of PP_i on the steady-state light production in the presence of L-luciferin was examined. Both at low (Figure 3c) and high ATP concentrations (results not shown) PP_i-ase (0.5 unit/ml) prevents the reactivation after the initial flash, indicating that reactivation and ampli-

Table 1 Effect of L-luciferin on kinetic constants of firefly luciferase determined at the time of the peak light production

L-Luciferin was added to the D-luciferin stock solution and subsequently diluted to give the indicated fractional concentration (%) of D-luciferin. The concentration of D-luciferin was varied between 1 and 200 μM, that of ATP between 5 and 1000 μM. Luciferase concentration was 5 nM. Values of the constants, expressed in μM, are given as means $\pm$ S.E.M. of three experiments.

	Addition of L-luciferin (% of the D-luciferin concentration)		
	0	1	5
$K_S^{\text{D-luciferin}}$	245 ± 24.9	97.8 ± 10.4	49.1 ± 6.43
K_m^{ATP}	33.9 ± 3.04	68.1 ± 6.68	79.3 ± 7.19
K_S^{ATP}	565 ± 57.6	901 ± 181	650 ± 107
$K_m^{\text{D-luciferin}}$	14.5 ± 1.11	9.15 ± 1.32	6.81 ± 0.83

fication are PP$_i$-dependent processes. PP$_i$ prevents the transient inhibition at high concentrations of ATP and the amplification at low ATP concentrations in a concentration-dependent manner. No transient was observed in experiments performed in the presence of PP$_i$ (1 μM) and L-luciferin (0.1–1 μM).

No modification of the enzyme activity was observed by incubating luciferase with L-luciferin alone or with a mixture of D- and L-luciferin. Premixing the enzyme with L-luciferin and ATP, however, induces a slowly increasing light production.

Light production from L-luciferin

Firefly luciferase can emit light even in the absence of D-luciferin. If L-luciferin is added to a mixture of enzyme and ATP a slow increase of light production can be detected that reaches a stable plateau after several minutes. In the presence of 30 μM ATP and 5 nM luciferase an increase of the L-luciferin concentration from 0.1 to 1 μM increases the half-time to reach maximal intensity from 5.2 to 7.8 min. The final light production reaches 46 % and 17 % respectively of the light production from the corresponding D-luciferin concentration. Increasing the enzyme concentration from 5 to 50 nM while reducing the ATP concentration from 30 to 3 μM does not affect the steady-state activity of D-luciferin catalysis. However, the half-time of the L-luciferin catalysis under these conditions is reduced to 1.7 min at all tested concentrations (0.1–1 μM). The maximal light production reaches 27 % or 18 % respectively compared with the catalysis in the presence of an equivalent concentration of D-luciferin.

The light production from the D-isomer increases with increasing luciferase concentrations. At high enzyme concentrations (i.e. 100 nM) the stable signal is replaced by an initial peak light production that declines to a stable activity after several minutes. Addition of PP$_i$ after 6 min of catalysis lowers the enzyme activity but also stabilizes the signal (Figure 4a, ○). Catalysis in the presence of PP$_i$-ase results in a reduced initial activity that is insensitive to the addition of PP$_i$ (Figure 4a, solid line). Preincubation of luciferase with PP$_i$ results in an activity similar to that observed in the presence of PP$_i$-ase, further addition of PP$_i$ inhibits the reaction in a concentration-dependent manner (results not shown).

In contrast, light production from L-luciferin is not increased by high enzyme concentrations, hence only a low activity can be observed. Addition of PP$_i$ after 6 min of catalysis does not affect this low intensity of light production (Figure 4b, ○). A similar signal is observed when luciferase is premixed with PP$_i$, and further addition of PP$_i$ after 6 min of catalysis has no effect on
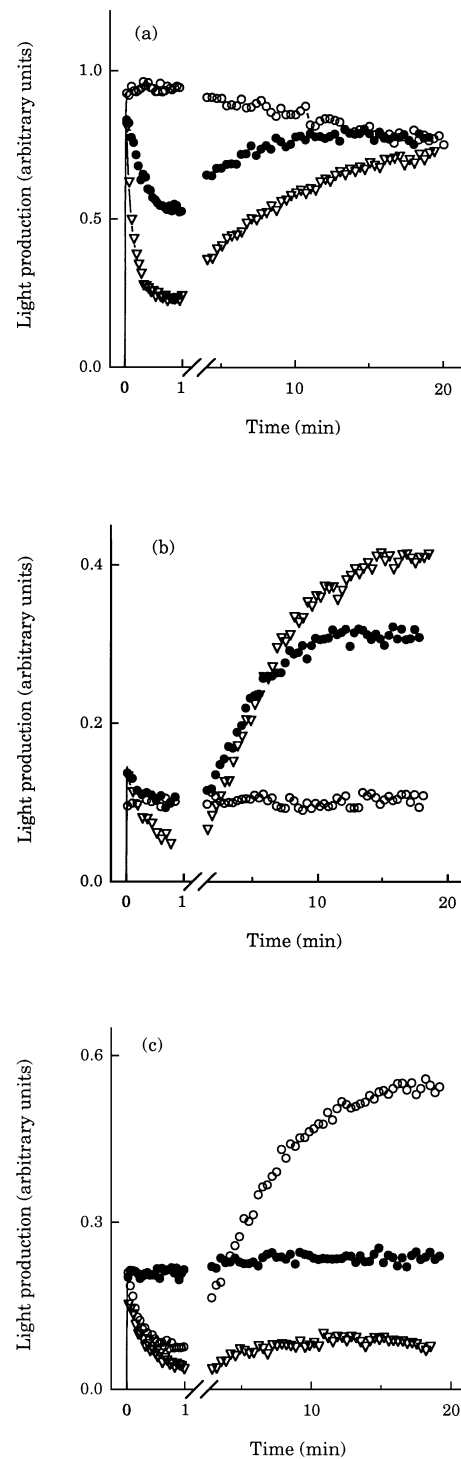
Figure 3 Effect of L-luciferin and PP$_i$ on steady-state light production of firefly luciferase

(**a**) Luciferase (5 nM) was premixed with ATP (30 μM). The reaction was started by the addition of D-luciferin (0.1 μM; ○); or by addition of a mixture of D-luciferin (0.1 μM) and L-luciferin (0.2 μM) (●); or by addition of a mixture of D-luciferin (0.1 μM) and L-luciferin (1 μM) (▽). (**b**) The corresponding experiment in the presence of 10 μM ATP. (**c**) The experiments were performed in the presence of 5 nM luciferase, 10 μM ATP, 0.1 μM D-luciferin and 1 μM L-luciferin. ○ indicates the light production in the absence of PP$_i$ or PP$_i$-ase. ● denotes the time course of light production in the presence of added PP$_i$ (1 μM). ▽ indicates the reaction in the presence of PP$_i$-ase (0.5 unit/ml).
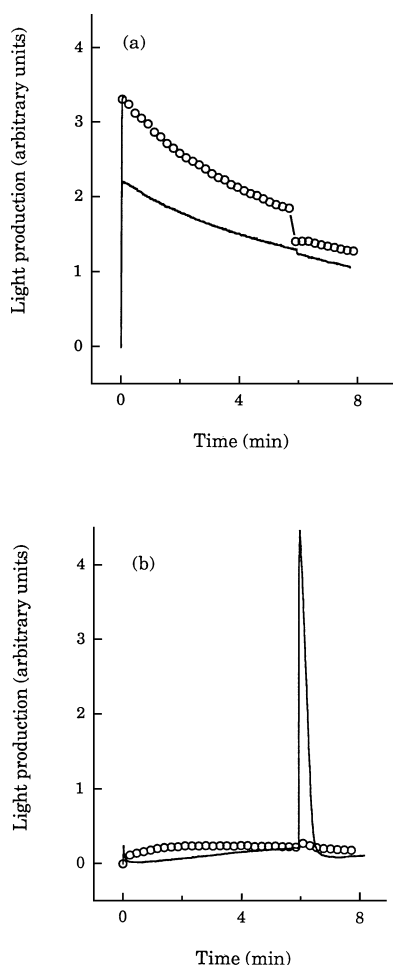
Figure 5   Repeated flashing of firefly luciferase induced by PP$_i$ in the presence of L-luciferin

The enzyme (0.1 μM) was premixed with ATP (100 μM) and PP$_i$-ase (0.2 unit/ml). The light production was recorded after the addition of L-luciferin (1 μM). (**a**) The mixture was allowed to equilibrate for 5 min before the addition of PP$_i$ (10 μl of a PP$_i$ stock solution of 500 μM). Additional injections of PP$_i$ aliquots were made after 6 and 7 min respectively. (**b**) A new sample was allowed to equilibrate with L-luciferin for 6 min before PP$_i$ was added. Additional injections of PP$_i$ were made after 12 and 18 min respectively.

Figure 4   Flash induction by PP$_i$: difference between D- and L-luciferin kinetics on stimulation with PP$_i$

(**a**) The reaction was started by addition of 0.2 μM D-luciferin to the enzyme solution (luciferase 0.1 μM, ATP 100 μM), PP$_i$ was added after 6 min (10 μl of a PP$_i$ stock solution of 500 μM) (○). The solid line denotes the light production of firefly luciferase in the presence of PP$_i$-ase (0.2 unit/ml), PP$_i$ was added as described above. (**b**) The corresponding experiment performed in the presence of 1 μM L-luciferin and in the absence of D-luciferin.

the enzyme activity (results not shown). If luciferase is premixed with PP$_i$-ase the activity remains low although the baseline is not completely stable but slowly increasing. Upon the addition of PP$_i$, however, the system emits a flash (Figure 4b, solid line).

When PP$_i$ is added shortly after the preceding flash (Figure 5a) the maximal peak light intensity is only a fraction of the first flash. However, if PP$_i$ is added at longer time intervals (Figure 5b) the system regains full responsiveness. The flash emitted after 18 min is not significantly lower than the initial flash emitted after 6 min ($P > 0.05$; $n = 3$). Since the concentration of PP$_i$ determines the peak height and duration, a relatively low PP$_i$-ase concentration was chosen to obtain a high flash intensity. Increasing the PP$_i$-ase concentration from 0.2 to 2 units/ml reduces the peak height by about 70 % of the activity observed in Figure 5(b).

DISCUSSION

The present work demonstrates light production from L-luciferin. So far attempts to detect light production from L-isomers of
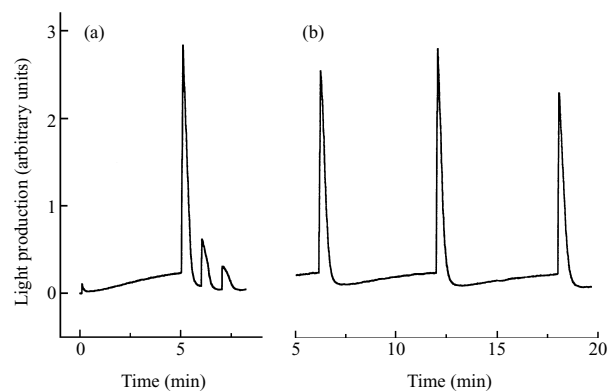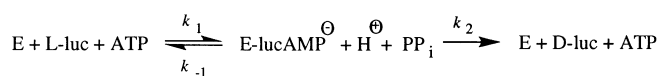
luciferin or its analogues have failed [12,15]. At high concentrations the L-isomer behaves as a competitive inhibitor with respect to D-luciferin which is in agreement with some of the previous observations [12,14] and ensures that the results obtained are properties of L-luciferin. Furthermore high concentrations of L-luciferin do not emit light or the light emission occurs after a considerable lag phase. Hence light production from L-luciferin cannot be observed in peak light analysis. Due to the slow onset of light production, monitoring over longer periods of time and the use of low L-luciferin concentrations are necessary. This may explain why previous attempts to detect light production from L-luciferin have been unsuccessful.

For a random bireactant mechanism it can be predicted that substrate contamination with a competitive inhibitor affects all kinetic constants. In the present case uncompetitive inhibition with respect to D-luciferin and mixed inhibition with respect to ATP ought to be expected. The prediction agrees with the observation for D-luciferin, but fails with respect to ATP where a competitive inhibition is instead observed. Obviously a more complex binding mechanism involving more binding sites for L-luciferin would meet the observation but the present data do not justify a specific modification of the reaction scheme.

The characteristics of the light production from L-luciferin indicate enzymic catalysis with some distinct differences compared with catalysis of the D-isomer. The half-time to reach maximal activity ranges from 1 to 8 min and is at least 200 times longer than the immediate onset of light production in the catalysis of D-luciferin (0.3 s). It has been proposed that proton abstraction at the carbon 4 position [16] and a large conformational change of the enzyme [17] initiate the oxidative decarboxylation of the luciferyl adenylate. The relatively slow increase to maximal light production (3–9 s) observed in kinetic studies with D-luciferin analogues was attributed to a retardation of one of these steps and was explained by further conformational changes of luciferase after binding the analogue [15]. Since it is known that L-luciferin does not react with oxygen [12] the final conformational change initiating decarboxylation may be excluded in the analysis of L-luciferin catalysis, but the participation of a slow proton abstraction in the observed slow increase of light production must be considered.

$$E + \text{L-luc} + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E-lucAMP}^{\ominus} + H^{\oplus} + PP_i \overset{k_2}{\longrightarrow} E + \text{D-luc} + ATP$$

Scheme 1 Proposed mechanism of L-luciferin racemization upon PP_i stimulation

It is assumed that the production of L-luciferyl adenylate from ATP and L-luciferin is fast compared with the slow deprotonation step (k_{-1}). For simplicity the production of L-luciferyl adenylate is omitted.

It is proposed here that in the absence of PP_i the L-luciferyl adenylate is deprotonated slowly and a luciferyl adenylate with a carbanion at the 4 position is accumulated (Scheme 1). This adenylate must be structurally different from the corresponding adenylate produced from the D-isomer since oxidative decarboxylation and hence light emission are not observed. Upon PP_i addition racemic luciferins are produced if the reversible proton abstraction is not stereoselective. As a consequence the light production from L-luciferin should reach about half the level obtained in the presence of the D-isomer which is in good agreement with the observed values. Furthermore, one has to assume that the luciferins are not released from the enzyme after racemization, since released D-luciferin would compete with excess free L-luciferin and no light emission should be observed. According to Scheme 1 the system must be free of PP_i to emit flashes in response to a PP_i addition. Since adenylation of L-luciferin releases PP_i, it may be expected that high concentrations of enzyme prevent accumulation of luciferyl adenylate due to a significant PP_i production. The weak light production in the catalysis of L-luciferin (Figure 4b, $\bigcirc$) may be interpreted as a continuous luciferin racemization. Since the deprotonation (k_{-1}) is slow the interval between two PP_i additions must be kept low as well. At high frequencies of PP_i stimulation the deprotonation of L-luciferyl adenylate becomes rate limiting, hence reducing the amount of D-luciferin produced and consequently the flash intensity.

A similar slow kinetic transient of luciferase with a half-time of several minutes was recently described [10]. The transient inhibition was precipitated by incubation of firefly luciferase with D-luciferin. Reactivation occurred in the absence of ATP and the initial activity was restored after approx. 20 min. In that paper it was proposed that binding of a second D-luciferin molecule induced the transient inhibition. However, later on an HPLC analysis of the D-luciferin batch used in that study [10] demonstrated the presence of an L-luciferin contamination (1%) and some unidentified substances (0.5%). Since L-luciferin fails to induce the transient inhibition in the presence of high concentrations of D-luciferin it is instead suggested that an as yet unidentified luciferin contamination with a high binding constant caused the transient inhibition. There is at present not enough information to decide whether a conformational change, as discussed originally, or a dark catalysis similar to that pertaining to L-luciferin, caused the observed transient.

Repeated flashing cannot occur in the presence of high concentrations of ATP and D-luciferin alone since the enzyme is inhibited after one flash due to the production of oxyluciferin, which is a strong competitive inhibitor with respect to D-luciferin [18]. In contrast, flash emission from L-luciferin does not imply substrate saturation since it is observed at low concentrations of L-luciferin. Repeated flashing according to Scheme 1 may have alternative explanations. Either the luciferase molecule participating in a flash becomes inhibited by oxyluciferin for a long time. In this case the L-luciferin concentration should be kept low

compared with the concentration of luciferase in order to avoid enzyme depletion. If, on the other hand, luciferase completes the catalytic cycle and liberates oxyluciferin the refractory phase between flashes may result not only from the slow deprotonation (k_{-1}) but also from liberation of oxyluciferin from the enzyme. As a consequence even saturating concentrations of L-luciferin should be possible with a concomitant increase in flash intensity. Since kinetics similar to Figure 4(b) can be observed by lowering the enzyme concentration to 5 nM (results not shown) the second alternative seems more plausible, especially since PP_i is known to liberate oxyluciferin from the enzyme [8,14].

It may be speculated whether L-luciferin participates in flash control *in vivo*. While not proven explicitly native luciferin may contain some L-luciferin. Attempts to purify native D-luciferin resulted either in racemic luciferin mixtures [19] or in luciferin preparations with lower activity in bioluminometric assays than the crude luciferin [20]. The pulsatile racemization of L-luciferin would allow the enzyme to effectively combine light production with a minimal oxyluciferin production. If the rapid equilibrium assumption for substrate binding is valid the K_i for L-luciferin represents a true dissociation constant. Hence L-luciferin binds approximately three times more strongly than the catalytically active D-isomer. This may indicate a participation of L-luciferin in the control of flashing *in vivo*.

In contrast to continuous ATP monitoring with perfused D-luciferin the system presented here would respond primarily to local PP_i changes. It may be useful in order to monitor intracellular events involved in cell activation, e.g. activation of adenylate cyclase by hormones or neurotransmitters leading to a concomitant liberation of cAMP and PP_i.

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