Regulatory effects of ATP and luciferin on firefly luciferase activity

Nicolas LEMBERT* and Lars-Åke IDAHL

Department of Histology and Cell Biology, Umeå University, S-90187 Umeå, Sweden

ATP and luciferin are not only substrates of firefly luciferase, but can, in addition, modulate its activity. High concentrations of luciferin induce a conformational change of the enzyme that temporarily reduces the catalytic rate. Re-activation takes approx. 20 min and is independent of variation in the concentration of enzyme or ATP, but lengthens with increasing luciferin concentration. High concentrations of albumin reduce this luciferin effect. The kinetic properties of firefly luciferase determined from initial rates and at steady state after 1 min of

catalysis have been analysed according to Michaelis–Menten kinetics. There is only one active site for each of the substrates. At steady state the K_m and V_{max} values for both substrates are reduced in an uncompetitive manner. Hyperbolic Lineweaver–Burk plots indicate an activation by ATP probably by binding to an allosteric site. A model is presented which incorporates luciferin induced de- and re-activation effects. Experimental conditions to avoid the regulatory effects of substrates during ATP monitoring are proposed.

INTRODUCTION

The light production of firefly luciferase is one of the most sensitive analytical tools for detection of ATP. With a detection limit in the femtomolar range it should be an ideal method for measuring intracellular ATP concentrations in living cells. At present two different models are used to describe the luciferase kinetics. The Rapid Equilibrium Random bireactant mechanism [1,2] can be applied to peak-light analysis. The post-flash decay is thought to result from a slow product release. The finding of a second binding site for MgATP led to an extension of this model. The peak and the low-level light production in the steady state have since then been ascribed to the action of two independent ATP sites [3]. According to this model a low-affinity site is inactivated after the peak, whereas a high-affinity site is responsible for the activity at steady state.

However, experiments performed in the presence of low ATP concentrations revealed a slow kinetic transient, whereas existing models predict a stable activity under these conditions. A mechanism that explains the observed transition is proposed and compared with the previous models.

MATERIALS AND METHODS

Chemicals

Firefly luciferase (EC 1.13.12.7), D-luciferin (luc), ATP and electrophoretically homogeneous, freeze-dried BSA were purchased from Boehringer (Mannheim, Germany). Hepes was obtained from Calbiochem (La Jolla, CA, U.S.A.). KOH (Suprapur) was from Merck (Darmstadt, Germany). KCl and MgCl₂ were of 'pro analysi' grade. Quartz-bidistilled water was used throughout. The reagent buffer used in all experiments consisted of 50 mM Hepes adjusted to pH 7.6 with KOH, containing 20 mM KCl and 5 mM MgCl₂. Albumin (0.01 %, w/v) was always included in order to stabilize the enzyme. All experiments were performed at room temperature.

Analytical procedure

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

of buffer containing enzyme and one of the substrates with a 1 ml plastic syringe into 7 µl of buffer with the second substrate placed in a polythene micro test tube (Milian Instruments S.A., Geneva, Switzerland) mounted in front of the photomultiplier. The signal from the fluorimeter was transferred to a Macintosh II fx computer (Apple Computer Inc., Cupertino, CA, U.S.A.) via an analog-to-digital converter (ACM2; Strawberry Tree, Sunnyvale, CA, U.S.A.) and analysed with the Workbench program from the same supplier.

The ATP concentration was varied between 0.2 and 500 µM, and that of the luc between 1 and 200 µM. In the course of one experiment both substrate concentrations were varied and the reaction rate (i.e. the light intensity) was determined at two time points, at the peak level and 60 s after the injection.

A Packard model 3310 Tri-Carb spectrometer was used to measure the light production in experiments at very low substrate or enzyme concentrations. The light production was detected in the repeat mode (five cycles/min). The signal was integrated over a period of 6 s.

Determination of kinetic parameters

Hyperbolic concentration–rate dependencies were analysed by least-square fits to the Michaelis–Menten equation using the Marquardt–Levenberg search algorithm. The resulting parameters K_{app} and V_{app} were used in secondary plots to determine K_m , K_s and V_{max} . Mean values were compared using independent Student's *t* test. These mathematical operations were carried out with the SigmaPlot 4.1 program (Jandel Scientific G.m.b.H., Erkrath, Germany) on Macintosh computers. To compensate for experimental variations in the enzyme activity, the rates in all experiments were normalized by dividing all values with the highest rate measured for the smallest concentration of the fixed substrate (1 µM luc or 5 µM ATP). This allows the graphic representation of mean values ± S.E.M. in Hanes or Lineweaver–Burk plots. Furthermore, the maximal rates determined from those plots can then be directly compared.

Discrimination between rival kinetic models

The kinetic simulation program KINSIM [4] combined with the automatic regression routine FITSIM [5] was used to obtain rate

Abbreviations used; luc, luciferin [p-(–)-2-(6'-hydroxybenzothiazolyl)-Δ²-thiazoline-4-carboxylic acid]; SSQ, sum of squares; Q², mean sum of squares.

* To whom correspondence should be addressed.

Scheme 1 Proposed mechanism of luc induced de- and re-activation of luciferase

and equilibrium constants according to the proposed model of re-activation (Scheme 1). These programs were executed on a VAX computer.

In FITSIM each point at a given time in the simulated progress curve is numerically compared with the corresponding time point in the experimental curve by calculating the summation of the squared differences (SSQ) between the two. In order to compare light production measured in volts with concentration values used in the regression calculation all values were normalized. The overall SSQ value between all the simulations and experimental progress curves is determined by eqn. (1):

$$SSQ = \sum_{s=1}^{n_s} w_s \left[\sum_{t=1}^{t-m} w_t (exp_t - cal_t)^2 \right] \quad (1)$$

where n_s is the number of data sets and m is the maximal simulation time. exp_t (light production) and cal_t (concentration of ternary complexes E-luc-ATP and E-luc₂-ATP) are the experimental and calculated points at the reaction time, t , for a given simulation parameter set, s . Unequal variance of the experimental data at time t can be compensated for by the weighting factor, w_t . Weighting by slope was selected in the present analysis to increase the fitting precision at the time of the post-flash decay. This option makes the weight of data proportional to the slope of the tangent. w_t is used to normalize each full time course to a common value to compensate for differences in number of data points.

A model with n data and p parameters has $(n-p)$ degrees of freedom and the mean sum of squares (Q^2) is determined by eqn. (2):

$$Q^2 = SSQ/(n-p) \quad (2)$$

According to Mannervik [6], a good model shows a random distribution of residuals around the zero level and a low Q^2 value. The residuals should be comparable with the experimental error. The simplest model fulfilling the above criteria was chosen as the most suitable mechanism.

RESULTS

Kinetic properties determined at the peak

The analyses after preincubation of luciferase with ATP result in plots typical for a random bireactant mechanism with the kinetic constants given in Table 1.

The calculated mean of the products of K_m^{ATP} and K_s^{luc} ($6423 \pm 588 \mu M^2$) is not significantly different ($P > 0.05$) from the calculated mean of K_m^{luc} and K_s^{ATP} ($4808 \pm 968 \mu M^2$).

Table 1 Catalytic properties of firefly luciferase determined at the time of peak light intensity

The reagent containing the enzyme was preincubated with ATP or luc for 30 min prior to injection of the second substrate. All experiments were carried out in triplicate and the results are presented as mean values (μM) $\pm$ S.E.M. The concentration of ATP ranged from 5 to 500 μM and that of luc from 1 to 200 μM . The luciferase concentration was 6 nM.

Parameter	Preincubation of luciferase with ...	Value (μM)	
		ATP	luc
K_m^{ATP}		48.5 ± 2.6	59.4 ± 5.0
K_m^{luc}		8.34 ± 1.02	$7.89 \pm 1.17^*$
K_s^{ATP}		603 ± 153	558 ± 110
K_s^{luc}		132.7 ± 11.8	$51.3 \pm 13.9^\dagger$

* In this case the concentration of luc as the fixed substrate ranged from 1 to 50 μM .

† $P < 0.05$ compared with preincubation with ATP.

Preincubation of the enzyme with luc reduces the luc dissociation constant by 60%, whereas the other kinetic constants remain unchanged. Still the mean values for the products are not significantly different (K_m^{ATP} and K_s^{luc} give $2907 \pm 588 \mu M^2$, K_m^{luc} and K_s^{ATP} give $4624 \pm 1507 \mu M^2$). Thus, at the given experimental precision, these results are in agreement with a random bireactant mechanism.

At higher luc concentrations ($> 50 \mu M$) an increasing substrate inhibition by luc can be detected. Experiments performed at very low ATP concentrations (0.2–5 μM) yielded essentially the same values as those shown in Table 1. There is only one detectable K_m value for each of the substrates.

The slow kinetic transient

Luciferase preincubated for 30 min with luc displays a relatively stable activity at low substrate concentrations (Figure 1, ○). However, in experiments started without preincubation as well as after preincubation of luciferase with ATP, there are pronounced changes in activity with time. There is an initial peak followed by a slow kinetic transient. This phenomenon can be detected with luc concentrations higher than 20 μM . The half-time of the transient increases with increasing luc concentrations, but shortens with increasing concentrations of albumin. Thus, at an albumin concentration of 0.1 mg/ml, the half-time changes from 1.8 min at 50 μM luc to 7.9 min at 500 μM luc. A 100-fold

Figure 1 Effect of substrate preincubation on the light production of firefly luciferase: light production of luciferase in the presence of low substrate concentrations

The enzyme was preincubated with 200 μM luc and the reaction was started by manual injection of 1 μM ATP (○). The continuous line denotes the corresponding experiment after preincubating the enzyme with ATP. (a) Time course of the luciferase reaction during the first 8 s after the initiation of the reaction. (b) Full time course of the reaction.

Table 2 Comparison between rival kinetic models

The variation of initial rates observed after preincubation of luciferase for different periods of time with luc was fitted to the proposed model of re-activation (Scheme 1) with the regression program FITSIM. Three models were examined by increasing the number of varied parameters. The goodness-of-fit is expressed as SSQ and Q^2 . Since preincubation times less than 20 s cannot be controlled precisely enough, those data points were taken from progress curves ($n = 3$). A total of 209 data points from seven experiments were used in the analysis. The non-varied (n.v.) parameters were set to zero. K_m and K_s values were taken from Table 1. Experimental conditions were: ATP, 1 μM ; luc, 200 μM ; luciferase, 5 nM; and albumin, 0.1 mg/ml.

No. of varied parameters ...	3	4	5
$10^4 \times \text{SSQ}$	24.7	6.58	6.76
$10^6 \times Q^2$	11.9	3.21	3.31
k_1 ($\text{M}^{-1} \cdot \text{s}^{-1}$)	8416 ± 174	9841 ± 91.1	9973 ± 90.7
k_{-1} (s^{-1})	n.v.	0.39 ± 0.01	0.39 ± 0.01
$k_2 \cdot 10^3 \times$ (s^{-1})	8.64 ± 0.51	4.32 ± 0.07	5.15 ± 0.08
$k_{-2} \cdot 10^4 \times$ (s^{-1})	n.v.	n.v.	2.47 ± 0.06
K_m^{ATP} (μM)	45.64 ± 0.82	37.93 ± 0.42	38.49 ± 0.37

increase in albumin reduces the half-time by almost 50% from 4.1 min at 0.1 mg/ml to 2.4 min at 10 mg/ml albumin at a luc concentration of 200 μM . Neither ATP (0.01–1 μM) nor luciferase (3 pM–10 nM) affect the half-time of the transient.

After 20 min of catalysis there is no difference in the steady-state activity, whether the enzyme has been preincubated with ATP or luc. Preincubation with luc for 15 s completely abolishes the peak, whereas the following transient is unchanged (results not shown). The initial rates obtained after addition of ATP to luciferase preincubated for various times with luc increase slowly and reach the initial activity after approx. 20 min.

This phenomenon can be explained by the mechanism shown in Scheme 1. According to Michaelis–Menten kinetics it is assumed that the initial light production corresponds to the concentration of ternary complexes. The shift in the luciferase activity is independent of catalysis, since it is a function of the

Figure 2 Transitional inactivation of luciferase by luc

The data points represent the normalized initial light production of luciferase preincubated for different periods of time with luc (200 μM) when started with ATP (1 μM). Since preincubation times less than 20 s could not be controlled precisely enough, those data points were taken from progress curves. The continuous line represents the ternary-complex concentration and was calculated as the least-squares fit according to Scheme 1 using the KINSIM and FITSIM programs.

contact time between enzyme and luc. In preincubations of luciferase with luc, the binary complex, E–luc, accumulates. Our model explains the inactivation of luciferase as the result of the binding of an additional luc molecule to form an inactive complex, $\text{E}^* \text{--} \text{luc}_2$. The slow transient is due to an isomerization of $\text{E}^* \text{--} \text{luc}_2$, leading to a catalytically active form.

Rate and equilibrium constants are obtained by fitting the initial rates to Scheme 1 using the KINSIM and FITSIM programs. Three rival models have been analysed. The simulation using four parameters gave the lowest SSQ and Q^2 and was thus chosen as the best-fitting model (Table 2). Figure 2 shows the variation of the initial light production as a function of the preincubation time with luc. The continuous line is the four-parameter simulation of Scheme 1. The distribution of residuals is plotted versus the predicted initial rates in Figure 3. There is an essentially random distribution of the values within the 95% confidence interval as determined from t statistics and the calculated experimental error.

Post-flash kinetics determined 1 min after the peak

A linearization of the rates determined 1 min after the peak is only possible at low substrate concentrations. Analysis of secondary plots after preincubation with luc yields a K_m for ATP of 6.1 μM and a K_m for luc of 2.7 μM . The V_{max} is reduced to 30% of the peak light activity. Analysis after preincubation with ATP shows a similar reduction of the K_m values, whereas the V_{max} is reduced to 13% of the peak value.

Lineweaver–Burk plots are curved near the ordinate at high substrate concentrations. Increasing luc concentrations inhibit the reaction, leading to upwardly curved plots. Increasing ATP concentrations activate the enzyme, and this results in hyperbolic plots (Figure 4). This phenomenon is common for both ATP and luc preincubations.

Figure 3 Error distribution between calculated and observed data from Figure 2

The continuous line shows the 95% confidence interval that was calculated from the experimental variance determined in replicate measurements. (a) Error distribution during the first 20 s of catalysis. (b) Error distribution during the slow transition.

Figure 4 Lineweaver-Burk plots of the light production 60 s after the initiation of the reaction

The enzyme was preincubated with luc. Mean values $\pm$ S.E.M. ($n = 3$) are shown. The fixed luc concentrations were 50 μM (○) or 200 μM (●).

DISCUSSION

It was reported previously [7] that the activity of luciferase during catalysis is reduced as a result of slow product release from the enzyme-product complex. The results presented here show that the catalytic properties of luciferase can be further influenced by their substrates, ATP and luc. The finding of a slow

kinetic transient makes it necessary to modify the Rapid Equilibrium Random bireactant mechanism commonly used in peak-light analysis. Scheme 1 shows the extension of that mechanism by a luc-induced conformational change.

According to the proposed mechanism, the binary complex E-luc is accumulated in luc preincubations. Binding of additional luc inactivates the E-luc complex. Hydrophobic substances such as detergents bind unspecifically to luciferase and induce conformational changes [8]. Depending on their chemical structure, stimulatory or inhibitory effects can be observed [8,9]. Since luc is very hydrophobic, the transient may be due to unspecific binding of luc to hydrophobic domains of the enzyme. $\text{E}^*\text{-luc}_2$ is inactive, since binding of additional luc molecules results in an unfavourable conformation that does not allow catalysis. However, there must be a reasonably fast equilibration between E-luc and $\text{E}^*\text{-luc}_2$ complexes, since the enzyme is not completely inhibited after the peak.

The kinetic simulation showed that the isomerization between $\text{E}^*\text{-luc}_2$ and E-luc₂ is mainly governed by a single rate constant, k_2 . The back reaction exerts only an insignificant influence on the formation of E-luc₂ from $\text{E}^*\text{-luc}_2$. Thus, after the transition is completed, E-luc is replaced by E-luc₂ and the stronger luc binding observed after preincubation with luc (Table 1) is consistent with this model.

The luc-induced conformational change is much slower than the substrate binding and is thus not observed in analyses of the initial rate when the enzyme has been preincubated with ATP. But once the enzyme has been in contact with luc, inhibition takes place. This explains the post-flash decay shown in Figure 1. Since k_1 is a second-order rate constant, the post-flash decay will only be observed at luc concentrations above a certain level.

According to Scheme 1, the rate constant k_3 makes the light production proportional to the concentration of ternary complexes. Since the analysis is based on normalized values, k_3 cannot be determined.

Albumin can bind to luciferase and stimulate the light production [8]. Thus high concentrations of albumin may prevent additional binding of luc to luciferase and reduce the transition induced by luc.

The analysis of the post-flash kinetics gives linear Lineweaver-Burk plots only at low substrate concentrations. V_{max} and K_m values for both substrates are reduced in an uncompetitive manner.

The reduced K_m value for ATP in post-flash measurements has been attributed to a second active site for ATP that becomes visible after the flash [3]. The model is based on the finding of an additional binding site for MgATP on the luciferase dimer. However the K_m value for luc is reduced in a manner similar to that of the K_m value for ATP. Thus the changed catalytic properties observed in post-flash kinetics are rather the result of the complex steady-state rate equation than of the action of a second active site for any of the substrates. Applying Michaelis-Menten kinetics in post-flash kinetics must change the meaning of K_m and V_{max} , since enzyme-product complexes are created during catalysis that are not present in the analysis of the peak light. The presence of a single active site for ATP is furthermore confirmed by the analysis of the initial rates at low ATP concentrations.

The observed substrate activation by ATP at high substrate concentrations indicates the presence of an allosteric binding site for ATP. The regulatory function of ATP, whereby binding of ATP promotes the product release from the E-P complex, was originally proposed by Rhodes and McElroy [7].

The stimulation of the post-flash light production by CoA [10] and CTP [11] has demonstrated the regulatory function of

nucleotides. Thus binding of nucleotides to the E-P complex may induce conformational changes that promote the product release. Since ATP binds to the E-P complex, the allosteric effect cannot be observed in the analysis of the initial rates or in the steady state at low substrate concentrations where the concentration of E-P is comparatively low.

The different $V_{\max}$ values obtained in post-flash measurements, depending on the order of substrate addition, are explained by the mechanism described in Scheme 1. Since the transition takes place in the presence of luc, but independent of catalysis, no E*-luc₂ complex is left after preincubation of the enzyme with luc for 30 min. In the opposite case, however, where the reaction is started by adding luc to ATP and luciferase a substantial amount of E*-luc₂ complex is formed which reduces the concentration of free enzyme and hence the catalytic rate.

The model presented here explains unstable luciferase kinetics observed at low concentrations of ATP and fairly high luc concentrations. Since bioluminometric assays are often performed with high concentrations of luc [12,13] to increase the total light production, the luc-induced transient during the first phase of monitoring must be carefully considered. Activation of post-flash measurements through an allosteric binding site for ATP further complicates monitoring, since a rise in the ATP concentration leads to non-hyperbolic concentration-rate dependencies.

The application of substrate analogues in monitoring experiments [14] demonstrated that the luciferase activity inside the cell is still sensitive towards allosteric regulators, limiting their usefulness in ATP-monitoring experiments [15]. The use of stimulatory substances has its advantage mostly in increasing the sensitivity of bioluminometric assays when using the firefly luciferase gene as a reporter [11]. Thus neither high luc concen-

trations nor the use of allosteric regulators are appropriate means of increasing light production during ATP monitoring. A stable steady-state luminescence may be achieved by pre-incubating the enzyme with luc. This prevents the initial burst of light and avoids the inactivation that follows. The apparent K_m value for ATP, furthermore, increases at low luc concentrations and extends the linear range during ATP monitoring.

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