RESEARCH COMMUNICATION

The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues

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The regeneration of an active semi-synthetic aequorin, from apoaequorin produced in cells and a coelenterazine analogue, is a key step in measuring Ca^{2+} in the cells. The relative rates of the regeneration of semi-synthetic aequorins from apoaequorin and 28 synthetic coelenterazine analogues were compared. The results indicated that the rate is strongly influenced by the analogues used. The regeneration of ordinary aequorin with normal coelenterazine was relatively fast (50% regeneration in 22 min), whereas the rates of regenerating semi-synthetic aequorins with coelenterazine analogues varied widely, and all were slower than that of regenerating ordinary aequorin, except for *e*-type coelenterazines (containing an extra ethano group). The regeneration with *e*-type coelenterazines was significantly faster, indicating the possible superiority of *e*-type analogues in the intracellular regeneration of aequorin, especially when an increased sensitivity to Ca^{2+} is needed.

INTRODUCTION

The photoprotein aequorin (mixtures of isoaequorins, M_r 20000–22000) has been widely used as a sensitive intracellular probe for Ca^{2+} for more than 20 years. Aequorin emits light in the presence of a trace amount of Ca^{2+}, decomposing itself into apoaequorin, coelenteramide and CO_2 [1]. Apoaequorin can be converted into the original aequorin by incubation with coelenterazine in the presence of O_2 [2], as shown below.

$$\text{Aequorin} \xrightarrow[\text{(fast)}]{Ca^{2+}} \text{apoaequorin} + \text{coelenteramide} + CO_2 + \text{light} \quad (1)$$

Scheme 1

$$\text{Apoaequorin} \xrightarrow[\text{(slow)}]{\text{Coelenterazine}+O_2} \text{aequorin} \quad (2)$$

Scheme 2

In the past several years, the usefulness of aequorin in Ca^{2+} measurement was further enhanced by two significant developments. The first was the successful production of several kinds of homogeneous recombinant aequorin, from recombinant apoaequorins and coelenterazine [3–7]. The second was the preparations of many different kinds of semi-synthetic aequorins, from apoaequorin and various synthetic analogues of coelenterazine [8–10]. The Ca^{2+}-sensitivity of semi-synthetic aequorins varies widely with the coelenterazine analogue used.

A new trend in the use of aequorin has begun recently [11–14], and it is becoming increasingly popular. In the new method, an apoaequorin is produced in cells by the utilization of the apoaequorin cDNA, then the apoaequorin is converted in the cells into a recombinant aequorin by the addition of coelenterazine which can permeate the cell membrane, and finally the aequorin produced in the cell can be used to study cellular Ca^{2+}. The method has a significant merit of avoiding the use of microinjection to introduce aequorin into cells, a technique which has been used previously, but is difficult and often impractical when the cells are small. It is expected that the new method will become more widely applicable by producing a semi-synthetic aequorin of suitable characteristics in the cell, by the use of a coelenterazine analogue in place of normal coelenterazine. Some experiments on this line were already reported [15]. In selecting a suitable coelenterazine analogue for this purpose, it is important to consider the ease of the regeneration reaction, in addition to the properties of the semi-synthetic aequorin to be formed. In the present paper, we report the rates of regeneration of semi-synthetic aequorins from apoaequorin and various analogues of coelenterazine.

EXPERIMENTAL

Materials

Natural aequorin was a part of the sample previously obtained [9] and called 'heterogeneous aequorin' in the present paper. The recombinant aequorin presently used was obtained as described previously [10] and called simply 'recombinant aequorin', although there are other recombinant aequorins. The analogues of coelenterazine were a part of the samples previously synthesized [9].

Preparation of apoaequorins

Aequorin dissolved in a pH 7.0 buffer ($A_{1cm,280} = 6.0$; approx. 2 mg/ml) was luminesced by dropwise addition of 10 mM calcium acetate at room temperature, so as to exhaust its light-emitting capacity completely in a period of about 30 min. The spent solution was cooled to 0 °C, and saturated with $(NH_4)_2SO_4$. After centrifugation, the pellet was dissolved in 10 mM Hepes/NaOH buffer, pH 7.6, containing 0.2 M KCl, 5 mM EDTA and 2 mM 2-mercaptoethanol, to make the final volume equal to that of the original aequorin solution (approx. 2 mg/ml). The apo-

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aequorin stock solution thus prepared was stored at −70 °C and used within 2 days; the storage of apoaequorin samples for an extended period of time resulted in apparent decrease in the rate of regeneration.

Regeneration reaction

The regeneration reaction was started by mixing 75 μl of an apoaequorin stock solution, 500 μl of the pH 7.6 buffer solution noted above, and 15 μl of 1 mM coelenterazine analogue in methanol ($A_{1cm,430}$ = 9.5), at 5 °C. The progress of regeneration was monitored by the periodical measurement of the total light-emitting capacity with an integrating light-meter, using a 5 μl portion of the reaction mixture in each measurement, as described previously [9].

RESULTS AND DISCUSSION

The time required for the 50% regeneration of semi-synthetic aequorins, from apoaequorin and 28 coelenterazine analogues, is shown in Table 1, together with the data for ordinary aequorin. The results clearly indicate that the rate of regeneration is strongly influenced by the coelenterazine analogues involved. The results also indicate that a modification of coelenterazine structure generally results in a decrease in the rate of regeneration, except for *e*-type coelenterazines (nos. 24–32) and *m(5)*-coelenterazine (no. 23), which are modified at position 5 of the imidazopyrazinone ring.

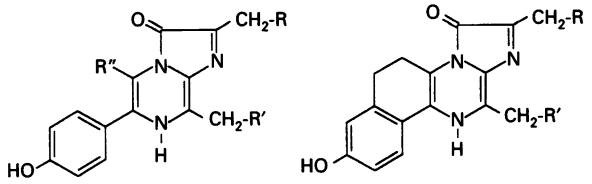
The periods of time required for 50% regeneration shown in Table 1 were measured at 5 °C. The effect of temperature on the regeneration rate was found to be relatively small; at 24 °C, the rates were about twice the rates at 5 °C. At 24 °C, however, the completion of regeneration was often not achieved under otherwise the same conditions, when the 50% regeneration time exceeded 1–2 h, presumably due to the auto-oxidation of the coelenterazine used. The intracellular regeneration is expected to be considerably slower if a lower concentration of a coelenterazine is used. Although a 50% regeneration of aequorin would be sufficient for most of the Ca^{2+} measurements, a full regeneration can be achieved, if needed, by extending the incubation time a few times longer than the 50%-regeneration time in the presence of a sufficient excess of a coelenterazine.

Various semi-synthetic aequorins having increased Ca^{2+}-sensitivities, such as *h*-, *f*-, *hcp*- and *fch*-aequorins (nos. 2, 3, 19 and 22), have been successfully used in the studies of cellular Ca^{2+}, by introducing them into the cells by microinjection. Those semi-synthetic aequorins are, however, regenerated rather slowly (50% in 95–210 min); thus appropriate attention should be paid if they are regenerated in cells.

The regeneration with *e*-coelenterazine (no. 24) was significantly faster than that with normal coelenterazine. All other *e*-type coelenterazines (nos. 25–32) were also considerably faster in the regeneration compared with the corresponding coelenterazine analogues not having the *e*-structure. The light-emission of *e*-type aequorins triggered by Ca^{2+} rises very rapidly to a peak intensity, a favourable characteristic in the measurement of cellular Ca^{2+} [8,9]. In addition, the light emitted is spectrally bimodal, with one peak at 400–410 nm and another at 440–475 nm, and the intensity ratio of the peaks is dependent on Ca^{2+} concentration, thus making it possible to determine the absolute concentration of Ca^{2+} based on the intensity ratio [8–10].

It is intriguing that the regeneration of *e*-type aequorins was inhibited by Ca^{2+}, whereas the regeneration reactions of all other types of aequorin were not. Thus a mixture of coelenterazine and apoaequorin left standing in the presence of Ca^{2+} shows a weak continuous luminescence, because any aequorin regenerated

Table 1 Relative rates of the regeneration of various semi-synthetic aequorins from apoaequorin and coelenterazine analogues



Coelenterazine *e*-Coelenterazine

R = C_6H_4OH-*p*; R′ = C_6H_5; R″ = H

The numbers and the prefixes correspond to those used in our previous papers [9,16,17]. A prefix summarily indicates the structural difference of a coelenterazine analogue compared with coelenterazine (nos. 2–23) or *e*-coelenterazine (nos. 25–32); it also indicates the semi-synthetic aequorin containing that analogue. R : *h*, deoxyphenyl; *f*, *p*-fluorophenyl; *f5*, pentafluorophenyl; *Cl*, *p*-chlorophenyl; *Br*, *p*-bromophenyl; and *n*, β-naphthyl. R′: *b*, n-butyl; *ip*, isopropyl; *cp*, cyclopentyl; *ch*, cyclohexyl; *m(8)*, phenylmethyl; and *f(8)*, *p*-fluorophenyl. R″: *m(5)*, methyl. Relative sensitivity to Ca^{2+} of an aequorin produced is graded into five classes, based on the relative luminescence intensities at pCa 7 previously reported [9,10,16,17], in which the luminescence intensity of recombinant aequorin made with unmodified coelenterazine was taken as 1.0: A (least sensitive), less than 1; B, 1–5; C, 5–20; D, 20–100; E (most sensitive), over 100.

No.	Prefix	Sensitivity to Ca^{2+}	Time for 50% regeneration (min)*
1 (coelenterazine)	none	B	22
2	*h-*	C	210
3	*f-*	C	140
5†	*f5-*	D	90
6	*Cl-*	A	24
7†	*Br-*	A	25
9	*n-*	A	300
10†	*b-*	C	60
11†	*ip-*	D	60
12	*cp-*	D	90
13	*ch-*	C	45
15†	*m(8)-*	C	45
16†	*f(8)-*	B	70
17	*fb-*	E	60
18†	*fip-*	E	40
19	*hcp-*	E	160
20†	*fcp-*	E	110
21	*hch-*	D	140
22	*fch-*	D	95
23	*m(5)-*	B	10
24 (*e*-coelenterazine)	*e-*	B	8
25†	*eh-*	C	24
26	*ef-*	D	10
27	*ech-*	C	13
28†	*ehch-*	C	60
29†	*efch-*	D	18
30†	*ecp-*	C	15
31†	*ehcp-*	D	80
32†	*efcp-*	E	40

* In 10 mM Hepes/NaOH buffer, pH 7.5, containing 0.2 M KCl, 5 mM EDTA and 2 mM 2-mercaptoethanol, at 5 °C. The amounts of apoaequorin and a coelenterazine analogue added in 0.59 ml of the reaction mixture were approx. 7.5 nmol and 15 nmol respectively.

† Natural heterogeneous apoaequorin was used; recombinant apoaequorin was used in the rest. The regeneration rates measured with these two apoaequorins were nearly equal when tested with nos. 1, 2, 3 and 22, suggesting that the rate is probably not significantly affected by the difference of apoaequorin.

(Scheme 2) instantly reacts with Ca^{2+} to emit light (Scheme 1), resulting in a gradual consumption of coelenterazine.

For a mixture of *e*-coelenterazine and apoaequorin, the regeneration reaction (Scheme 2) was completed only at Ca^{2+} concentrations less than 0.1 μM. At Ca^{2+} concentrations between 0.1 and 10 μM, the regeneration (Scheme 2) took place normally, but the *e*-aequorin regenerated instantly reacted with the existing Ca^{2+} to cause light emission (Scheme 2) whose intensity is dependent on both the amount of *e*-aequorin regenerated and the concentration of Ca^{2+} in the solvent; at 1 μM Ca^{2+}, the rates of the regeneration reaction and the luminescence reaction were nearly equal. The inhibition of regeneration by Ca^{2+} began at about 10 μM Ca^{2+}, and became significant at 100 μM Ca^{2+}. At Ca^{2+} concentrations greater than 1 mM the regeneration was completely inhibited; thus continuous luminescence, as seen with coelenterazine analogues other than *e*-type, did not occur. Based on the rates of regeneration (Table 1) and the various data previously reported [9,16,17], the maximum free Ca^{2+} concentration allowable in intracellular regeneration is estimated to be about 0.2 μM for *e*-aequorin, and 20 nM for *efcp*-aequorin (an *e*-type aequorin of the highest Ca^{2+}-sensitivity); possibly the concentration limit can be slightly increased by using a sufficient excess of the *e*-type coelenterazine to make up the loss being caused by the continuous luminescence.

In intracellular regeneration of aequorins, it is important that a coelenterazine chosen is introduced into cells in a sufficient concentration, without a significant loss caused by auto-oxidation. It has been already shown that coelenterazine and some of the analogues (*h*, *ip* and *e*-type) are sufficiently permeant through various cell membranes [11–15], although the permeability may vary considerably with different types of membrane, as well as by the substitution groups of the analogues. Similar permeabilities are highly likely, however, for *e*-coelenterazine and normal coelenterazine, or for an *e*-type coelenterazine analogue and a corresponding analogue without *e*-type structure, because of their similar molecular structure. Concerning the auto-oxidation, all coelenterazines are unstable even in their solid states. In solutions, they are significantly oxidized in 2–3 h, at 0–25 °C. Some analogues such as *h*-type coelenterazines are oxidized 2–3 times faster than normal coelenterazine and *e*-coelenterazine. Fortunately, however, coelenterazines appear to be more stable in cells than in open solutions. Thus it would be extremely important to minimize the auto-oxidation of coelenterazines that occurs before the start of experiments involving cells.

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Conclusion

For the intracellular regeneration of aequorin, the use of normal coelenterazine is a reasonable choice as far as the Ca^{2+}-sensitivity and other properties of ordinary aequorin are suitable. When a faster regeneration or a higher sensitivity (plus a faster response) to Ca^{2+} is needed, the use of an *e*-type coelenterazine, such as *e-*, *ech-*, *ecp-*, *ef-*, *efch-* and *efcp*-coelenterazine, should be considered.

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