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Resistivity to denaturation of the apoprotein of aequorin and reconstitution of the luminescent photoprotein from the partially denatured apoprotein

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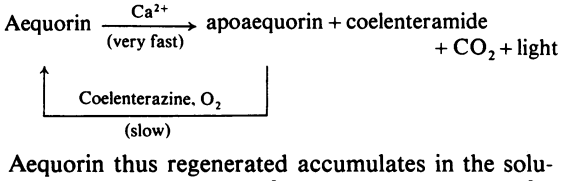
Although native aequorin is highly susceptible to inactivation, apoaequorin is highly resistant to various processes of denaturation. Apoaequorin was inactivated only partially at a temperature of 95°C or by treatments with 6 M-urea, 4 M-guanidine hydrochloride, 1 M-HCl and 1 M-NaOH. It was nearly completely inactivated in 85% ethanol or by heating at 95°C in 2 M-$(NH_4)_2SO_4$, but over 50% of apoaequorin activity was restored in both cases merely by dissolving the coagulated protein in 4 M-guanidine hydrochloride. In the reconstitution of aequorin, partially inactivated apoaequorin yielded more aequorin than expected from the activity of the partially inactivated apoaequorin used, suggesting that the process of reconstitution promotes the renaturation of denatured apoaequorin.

The photoprotein aequorin emits light when Ca^{2+} is added (Shimomura *et al.*, 1962; Blinks *et al.*, 1976). In this reaction, the active group of aequorin, i.e. a peroxide of coelenterazine (Shimomura & Johnson, 1975*a*, 1978), decomposes into coelenteramide plus CO_2, and these products easily dissociate from the protein part of aequorin, i.e., apoaequorin. Apoaequorin can be converted into active aequorin by incubation with coelenterazine in the presence of oxygen (Shimomura & Johnson, 1975*b*) as shown in the following scheme:



Aequorin thus regenerated accumulates in the solution in the absence of Ca^{2+}. In the presence of Ca^{2+}, however, the result is a spontaneous weak luminescence which lasts until all coelenterazine is consumed. In such a circumstance, apoaequorin acts as an enzyme catalysing the luminescent oxidation of coelenterazine. No method has been previously reported in restoring luminescent activity to a sample of inactivated aequorin, except when aequorin was deactivated through the process of Ca^{2+}-triggered luminescent reaction.

The use of aequorin luminescence in physiological studies has been popular in recent years, particularly in the monitoring of Ca^{2+} in single cells (see Ashley & Campbell, 1979). Despite this fact, the availability of aequorin is severely limited; commercial aequorin presently available is unsuitable for most research applications because of insufficient purity. The alternative of extracting and purifying the photoprotein oneself from the jellyfish *Aequorea* is not an easy task, mainly owing to the instability of the active substance. Also, the procedure is both expensive and time-consuming.

The unusual stability of apoaequorin reported in the present paper, combined with the fact that coelenterazine can be routinely synthesized (Inoue *et al.*, 1975), would provide the basis of a new, simpler way to prepare aequorin, by extracting and purifying apoaequorin under such conditions that most other proteins and enzymes will become denatured and inactivated, followed by the conversion of apoaequorin into aequorin.

Materials and methods

Coelenterazine, i.e., 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one, was a gift from Dr. S. Inoue, Meijo University, Nagoya, Japan. Guanidine hydrochloride, urea and $(NH_4)_2SO_4$ were Ultra Pure reagents from Schwarz–Mann. Tris, EDTA and Mops (4-morpholinepropanesulphonic acid) were from Sigma, and Bicine [*NN*-bis-(2-hydroxyethyl)glycine] was from Boehringer-Mannheim. Dimethyl malonate, diethyl

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malonate (both from Aldrich), and ethanol were redistilled before use.

Aequorin was extracted and purified as reported previously (Shimomura & Johnson, 1969, 1976). The concentration of aequorin was determined by absorbance at 280nm ($A^{1\%}_{1cm} = 27.0$) or by light-emitting capacity when Ca^{2+} is added (4.5×10^{15} quanta/mg at 25°C; Shimomura & Johnson, 1969).

Apoaequorin was prepared by allowing aequorin to luminesce in 2mM-$CaCl_2$/10mM-Tris/HCl, pH7.5. To separate apoaequorin from coelenteramide, 0.1vol. of 50mM-EDTA was added to the solution to chelate the existing Ca^{2+} and the mixture was passed through a small column of Sephadex G-25 (fine grade) (Pharmacia Fine Chemicals) prepared with 10mM-Tris/HCl buffer, pH7.5, containing 0.5M-NaCl and 1mM-EDTA. Apoaequorin was eluted in the void volume, whereas coelenteramide was adsorbed on the column. The concentration of apoaequorin was determined by A_{280} ($A^{1\%}_{1cm} = 18.0$; Shimomura & Johnson, 1969) or from the amount of aequorin initially used.

The activity of apoaequorin in catalysing the luminescent oxidation of coelenterazine was assayed by the intensity of luminescence emitted from 2ml of assay mixture, which consisted of 0.01–0.3ml of apoaequorin solution, 10μl of methanolic 0.2mM-coelenterazine, and 10mM-Tris/HCl buffer, pH7.3–7.5, containing 0.5M-NaCl, 2mM-$CaCl_2$ and 2mM-2-mercaptoethanol, at 23–25°C. The intensity of light was nearly constant for more than 30min.

Regeneration of active aequorin from apoaequorin was performed by adding 10μl of methanolic 0.6mM-coelenterazine to 0.5ml of 10mM-Tris/HCl, pH7.5, containing 1mM-EDTA, 5mM-2-mercaptoethanol and apoaequorin (less than 120μg). The progress of regeneration was monitored by Ca^{2+}-triggered luminescence obtainable from 10μl fractions of the reaction mixture. After 12h at 4°C, the amount of regenerated aequorin did not increase any further under the conditions involved.

Results and discussion

The ability of apoaequorin to catalyse the bioluminescent oxidation of coelenterazine in the presence of Ca^{2+} was found to be unexpectedly stable under various conditions for denaturing proteins. Representative results are shown in Table 1.

Apoaequorin activity was scarcely affected at 50°C in a pH7.3 buffer containing 0.5M-NaCl, and it was still not completely inactivated after heating at 95°C for 1h. The results of Expts. 1–5 (Table 1) indicate that the kinetics of inactivation involves two phases at least, namely a fast inactivation, which takes place during the first few minutes, and a slow inactivation thereafter. The extent of inactivation after heating at 95°C for 1h varied markedly with the pH of the solution, and the inactivation was minimal over the pH range 7–8 (Fig. 1). Treatment with 6M-urea or with 4M-guanidine hydrochloride resulted in only relatively slight inactivation (Expts. 9 and 10). On the other hand, heating in 2M-$(NH_4)_2SO_4$ and treatment with 85% ethanol resulted in the coagulation of the protein accompanied by almost complete loss of activity (Expts. 6 and 15), although over 50% of original apoaequorin activity was instantly recovered in the both cases when the coagulated protein was dissolved in 4M-guanidine hydrochloride, pH7.5.

The extent of inactivation of apoaequorin by heat was dependent on the concentration of apoaequorin, giving greater inactivation when apoaequorin was more diluted, as shown in Fig. 2. This evidence is intriguing in conjunction with the concentration–activity relationship of native apoaequorin shown in the same Figure, in which the activity is directly proportional to the concentration of apoaequorin (i.e., the specific activity is constant) for the concentration range below 10μg/ml, but the specific activity progressively declines at higher concentrations. The concentration effects noted above could be largely due to increased association or binding between apoaequorin molecules at higher concentrations, taking place in such a manner that the catalytic centre of the apoaequorin molecule becomes hindered and protected.

The concentration of apoaequorin also affected the yield of active aequorin regenerated from apoaequorin (Fig. 2). In this instance the regeneration was virtually quantitative for the concentrations of apoaequorin over 100μg/ml, but the yield fell to 40% when the concentration was 2μg/ml.

Apoaequorin treated with 4M-guanidine hydrochloride at 24°C for 10min retained 55% activity (Expt. 10, Table 1). When this product was subjected to the regenerating reaction after removing guanidine hydrochloride by dialysis, the yield of aequorin was over 85% (on the basis of the amount of initial apoaequorin). Apoaequorin heated at 95°C for 30min in 4M-guanidine hydrochloride, then left at room temperature for 3 days, retained no detectable activity, but the yield of regenerated active aequorin from this sample was 25% (on the basis of the amount of initial apoaequorin). Likewise, the yields of regenerated aequorin actually obtained from heat-treated apoaequorin samples (such as in Expts. 2–5, Table 1) were always considerably higher than the yields expected from the activities remaining in those heat-treated samples. These results seem to indicate that the process of regeneration promotes the renaturation of partially denatured apoaequorin.

The luminescence reaction of coelenterazine catalysed by apoaequorin in the presence of Ca^{2+} was activated by three categories of organic solvent that

Table 1. *Inactivation of apoaequorin under various conditions*

Apoaequorin (20 μg) was incubated in 0.3 ml of solvent, then activity was measured with the whole sample, except for Expts. 9–11 (assayed with 20 μl), Expts. 12 and 13 (neutralized), and Expts. 14 and 15 (ethanol removed under reduced pressure).

Expt. no.	Solvent*	Incubation conditions	Activity remaining (%)†
1	pH 7.3 buffer	None	100
2	pH 7.3 buffer	95°C, 2 min	75
3	pH 7.3 buffer	95°C, 5 min	65
4	pH 7.3 buffer	95°C, 15 min	40
5	pH 7.3 buffer	95°C, 1 h	21
6	2 M-$(NH_4)_2SO_4$ in pH 7.3 buffer	95°C, 2 min	0.2
7	2 M-NaCl in pH 7.3 buffer	95°C, 2 min	30
8	pH 7.3 buffer without NaCl	95°C, 2 min	30
9	6 M-Urea in pH 7.3 buffer	24°C, 10 min	92
10	4 M-Guanidine in pH 7.3 buffer	24°C, 10 min	55
11	4 M-Guanidine in pH 7.3 buffer	95°C, 30 min	7
12	1 M-HCl	24°C, 5 min	55
13	1 M-NaOH	24°C, 5 min	60
14	60% (v/v) Ethanol	24°C, 5 min	75
15	85% (v/v) Ethanol	24°C, 5 min	1

* All pH 7.3 buffers contained 10 mM-Tris/HCl, 0.5 M-NaCl and 2 mM-2-mercaptoethanol, except as noted.
† Catalysis of the luminescent reaction of coelenterazine in the presence of oxygen and Ca^{2+}.

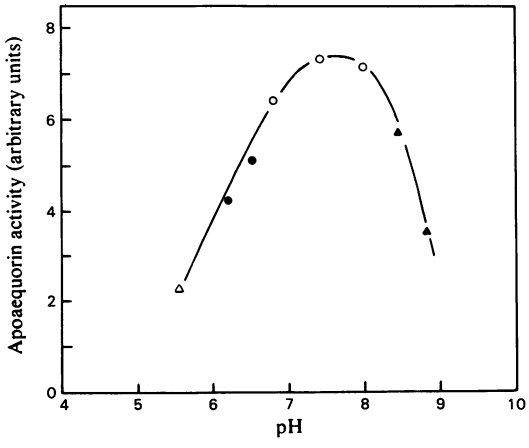

Fig. 1. *Influence of pH on the activity of apoaequorin*
Apoaequorin (21 μg) was first heated at 95°C for 1 h in 0.3 ml of buffer solution at various pH values, then its activity in the solution was assayed. The buffer solutions contained 10 mM-sodium acetate (△), -sodium phosphate (●), -Mops (○) or -Bicine (▲), in addition to 0.5 M-NaCl.

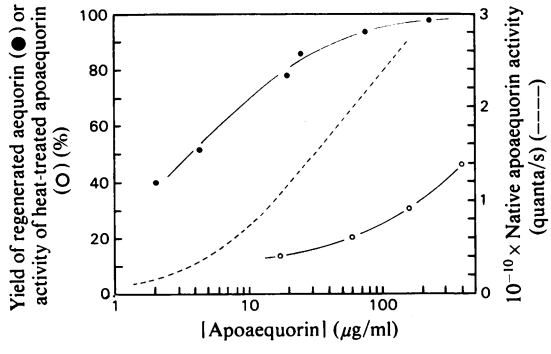

Fig. 2. *Effect of the concentration of apoaequorin on the yield* (%) *of regenerated active aequorin after 12 h at 4°C* (●), *on the recovery* (%) *of apoaequorin activity after heating at 95°C for 1 h in 0.3 ml of 10 mM-Tris/HCl, pH 7.5, containing 0.5 M-NaCl and 3 mM-2-mercaptoethanol* (○), *and on the activity of native apoaequorin* (----)
The concentrations of apoaequorin shown on the abscissa are for regeneration mixture (0.5 ml), heat-treated solution (0.3 ml) and the assay mixture of apoaequorin activity (2 ml) respectively.

are seemingly unrelated functionally: namely, acetonitrile, methyl acetate and ethyl acetate, and dimethyl malonate and diethyl malonate (Fig. 3). The effect of the malonates was strongest, giving an 8-fold activation under optimum conditions. In the absence of Ca^{2+}, the rate of regeneration and the final yield of active aequorin were both unaffected by these chemicals, thus suggesting either that Ca^{2+} is needed in the mechanism of activation, or, possibly, that aequorin is not an actual intermediate in the luminescent oxidation of coelenterazine catalysed by apoaequorin.

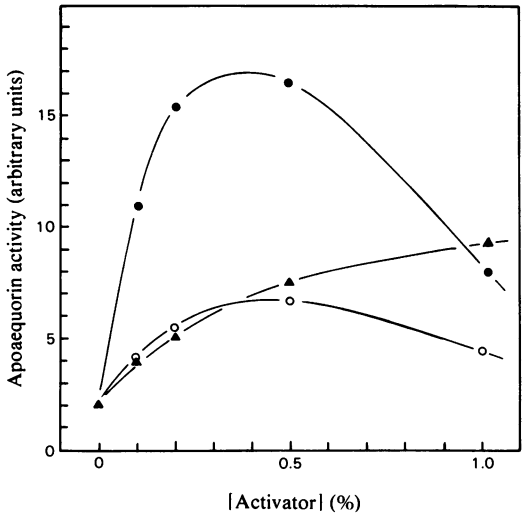

Fig. 3. *Relationship between apoaequorin activity and the concentration of activators*

●, Diethyl malonate; ○, ethyl acetate; and ▲, acetonitrile. Standard assay conditions were used, with 7 μg of apoaequorin.

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References

Ashley, C. C. & Campbell, A. K. (eds.) (1979) *Detection and Measurement of Free Ca^{2+} in Cells*, Elsevier/North-Holland Biomedical Press, Amsterdam

Blinks, J. R., Prendergast, F. G. & Allen, D. G. (1976) *Pharmacol. Rev.* **28**, 1–93

Inoue, S., Sugiura, S., Kakoi, H., Hashizume, K., Goto, T., & Iio, H. (1975) *Chem. Lett.* 141–144

Shimomura, O. & Johnson, F. H. (1969) *Biochemistry* **8**, 3991–3997

Shimomura, O. & Johnson, F. H. (1975*a*) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1546–1549

Shimomura, O. & Johnson, F. H. (1975*b*) *Nature (London)* **256**, 236–238

Shimomura, O. & Johnson, F. H. (1976) *Soc. Exp. Biol. Symp.* **30**, 41–54

Shimomura, O. & Johnson, F. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2611–2615

Shimomura, O., Johnson, F. H. & Saiga, Y. (1962) *J. Cell. Comp. Physiol.* **59**, 223–240