Volume 301, number 2, 197–201 FEBS 10945 April 1992

Two excited states in aequorin bioluminescence induced by tryptophan modification

Yoshihiro Ohmiya[a], Mamoru Ohashi[b] and Frederick I. Tsuji[a,c]

[a]*Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan,* [b]*Department of Applied Physics and Chemistry, University of Electro-Communications, Chofu, Tokyo 182, Japan* and [c]*Marine Biology Research Division, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA*

Received 7 February 1992

The Ca^{2+}-activated photoprotein, aequorin, contains six tryptophan residues and has a bioluminescence emission maximum at 465 nm. On converting the six tryptophan residues to phenylalanine, the mutant aequorins exhibited varied luminescence activities and spectra, but one mutant, with tryptophan-86 replaced by phenylalanine, gave a bimodal emission spectrum, with maxima at 455 nm and 400 nm. This result suggests that tryptophan-86 may be importantly involved in the generation of the product excited state during aequorin bioluminescence.

Photoprotein; Ca^{2+}-binding protein; Coelenterazine; Coelenteramide; Emission spectrum; Excited state

1. INTRODUCTION

Aequorin is a Ca^{2+}-binding photoprotein found in the jellyfish, *Aequorea victoria*, which emits light by an intramolecular reaction in the presence of Ca^{2+} [1–3]. The protein consists of a complex of apoaequorin (apoprotein), coelenterazine (organic substrate, M.W. 423), and molecular oxygen. Apoaequorin is made up of 189 amino-acid residues in a single polypeptide chain (M_r 21,400) with three EF-hand structures (Ca^{2+}-binding sites) [4,5]. The binding of Ca^{2+} converts the protein to a luciferase, which then catalyzes the oxidation of coelenterazine by the bound oxygen. The products of the reaction are light (λ_{max}=470 nm), CO_2, and coelenteramide (the oxidized product of coelenterazine). The excited state coelenteramide bound to apoaequorin is the emitter in the reaction [6]. Aequorin may be regenerated from apoaequorin by incubation with coelenterazine, dissolved oxygen, EDTA, and 2-mercaptoethanol [7].

As a Ca^{2+}-binding protein, the primary structure of aequorin is unusual in that it contains the amino-acid residues tryptophan, cysteine, and histidine, which are not commonly found in Ca^{2+}-binding proteins [5]. A previous study has shown that the cysteine residues may be involved in the regeneration of aequorin from apoaequorin [8], but the function of the other two residues is unknown. In this study, the six tryptophan residues were replaced by the close analogue phenylalanine, yielding mutant aequorins AQW12F, AQW79F, AQW86F, AQW108F, AQW129F, and AQW173F. One of the mutants, AQW86F, showed a striking change with a shift towards 400 nm in its bioluminescence emission maximum. This observation suggests that tryptophan-86 may play an important role in generating the product excited state in aequorin bioluminescence.

Correspondence address: F.I. Tsuji, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan. Fax: (81) (6) 872 4818.

2. MATERIAL AND METHODS

2.1. *Enzymes and chemicals*

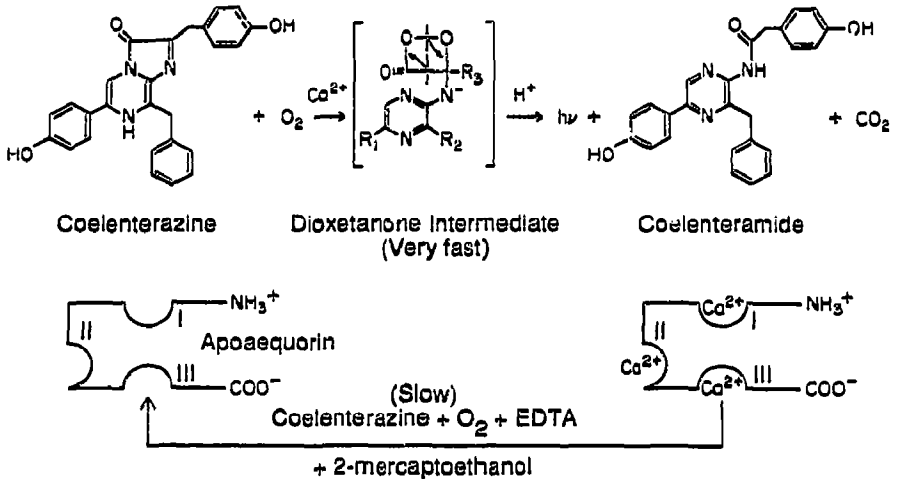
Restriction enzymes, *Escherichia coli* T4 DNA ligase and *Taq* polymerase were obtained from Takara Shuzo (Kyoto, Japan). Radiolabeled compounds were purchased from Amersham and DEAE-Cellulofine A500 was from Chisso Corp. (Yokohama, Japan). Oligonucleotides were synthesized by the phosphoramidite method [9] using an Applied Biosystems (Foster City, CA) Model 380A DNA synthesizer. Coelenterazine and coelenteramide analogues were chemically synthesized [10]. All other chemicals were of the highest grade commercially available.

2.2. *Bacterial strain and plasmid*

The bacterial strain was *E. coli* D1210 and the plasmid was the previously described piP-HE fused to the *ompA* secretion peptide coding sequence under the control of the *lpp* promoter and *lac* operator [11].

2.3. *Modification of tryptophan residues*

Tryptophan residues were replaced with phenylalanine residues by site directed mutagenesis using the polymerase chain reaction (PCR) [12,13] in which specific base substitutions were introduced as a mismatch between a PCR primer and the target sequence. Besides wild-type (piP-HE) apoaequorin (apoAQ), six mutant apoaequorins were prepared: apoAQW12F, apoAQW79F, apoAQW86F, apoAQW108F, apoAQW129F, and apoAQW173F. Each cDNA was sequenced to confirm the presence of the mutation by using a modified dideoxynucleic acid sequencing method [14]. Other molecular biology procedures were according to Sambrook et al. [15].



Scheme I

2.4. *Growth of bacteria and purification of recombinant apoaequorin*

Transformed *E. coli* D1210 was grown overnight at 30°C in 3 ml of LB medium, containing 50 μg/ml of ampicillin. The overnight culture was transferred to 25 ml of fresh LB medium, containing 50 μg/ml of ampicillin and the culture was incubated at 30°C with shaking for 3 h. The culture was finally transferred to 1000 ml of fresh M9CA medium containing 50 μg/ml of ampicillin and incubated at 37°C with shaking for 15 h. The secreted recombinant apoaequorin was purified from the culture medium by acid precipitation and column chromatography using DEAE-Cellulofine A500 [11]. The purified apoaequorin gave a single band on SDS-PAGE (12.5%) under reducing conditions and the yield was 0.5–30 mg of >95% pure apoaequorin per liter of culture medium.

2.5. *Regeneration of aequorin and assay for activity*

The purified apoaequorin was dissolved in 30 mM Tris-HCl, pH 7.60/10 mM EDTA at a concentration of 200 ng/ml. 200 μl of this solution was mixed with 2 μg of coelenterazine or coelenterazine analogue (1 μg/μl in absolute methanol) and 2 μl of 2-mercaptoethanol [11]. After standing in an ice-bath for 6 h, 50 μl of the incubation mixture was transferred to a glass reaction cell and injected with 1.5 ml of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60. The initial maximal light intensity was read with a Labo Science (Tokyo) Model TD-8000 photometer, calibrated with a carbon-14 light standard [16]. The initial maximal light intensity was converted to quanta per second to serve as a measure of activity. Bioluminescence emission spectra were measured with a Model I-MCPD multichannel photo-detection system (Otsuka Electronics, Osaka, Japan) under conditions described in the figure legend. The spectra were taken at the peak height of light emission.

2.6. *CD spectra of apoaequorin*

CD spectra were measured with a Jasco (Tokyo) Model J-600 spectropolarimeter using a cell with a path length of 0.1 cm. The protein concentration was 0.10 mg/ml in 30 mM Tris-HCl/10 mM EDTA, pH 7.6, estimated from the extinction coefficient at 280 nm, $E_{1\%,1.0cm}$= 18.0 [17]. All measurements were made at 4°C and each spectrum represents the average of at least three scans.

3. RESULTS AND DISCUSSION

Fig. 1 shows the chemical structures of coelenterazine and three analogues used to regenerate apoAQ, apoAQW12F, apoAQW79F, apoAQW86F, apoAQW108F, apoAQW129F, and apoAQW173F into their respective aequorins. Table I lists the bioluminescence activities and emission maxima of the regenerated aequorins. All of the mutant aequorins, except AQW79F, had lower activities than wild-type (piP-HE) aequorin (AQ). The activities ranged from a high of 124.4 for AQW79F to lows of <0.1 for AQW108F and AQW173F. The flash patterns of the Ca^{2+}-triggered aequorin emissions were nearly the same. Using coelenterazine as a substrate, the one-half rise time for AQ was 0.07 compared to values of 0.05 s to 0.12 s for the others, whereas the one-half decay time was 0.47 s for AQ and varied from 0.17 s for AQW173F to 0.81 s for AQW86F. Using coelenterazine as substrate, AQ, AQW12F, AQW108F, AQW129F, and AQW173F gave emission maxima of 465 nm, whereas AQW79F had a maximum at 460 nm and AQW86F had a maximum at 455 nm with a shoulder at 400 nm (Table I and Fig. 2A). The bioluminescence emission spectra for AQ and AQW86F regenerated with coelenterazine are shown in Fig. 2A and those for AQ and AQW86F regenerated with Compound b are shown in Fig. 2B. All AQW86Fs produced by regenerating with coelenterazine and the three analogues gave emission maxima that were shifted towards 400 nm.

The CD spectra of the apoaequorins following tryptophan modification were also measured to detect changes in secondary structure (Fig. 3). In each panel, the dashed line represents the CD spectrum of apoAQ and the solid line that of the mutant apoaequorin. The CD spectrum of AQ shows a typical protein secondary structure of α-helix and β-sheet. The CD spectrum of apoAQW12F indicates decreasing α-helix and possibly increasing random coil or β-sheet. The CD of apoAQW79F (Fig. 3B) shows a light change in secondary structure accompanied by a significant increase in bioluminescence activity (Table I). The CD of apoAQW86F (Fig. 3F), on the other hand, shows a

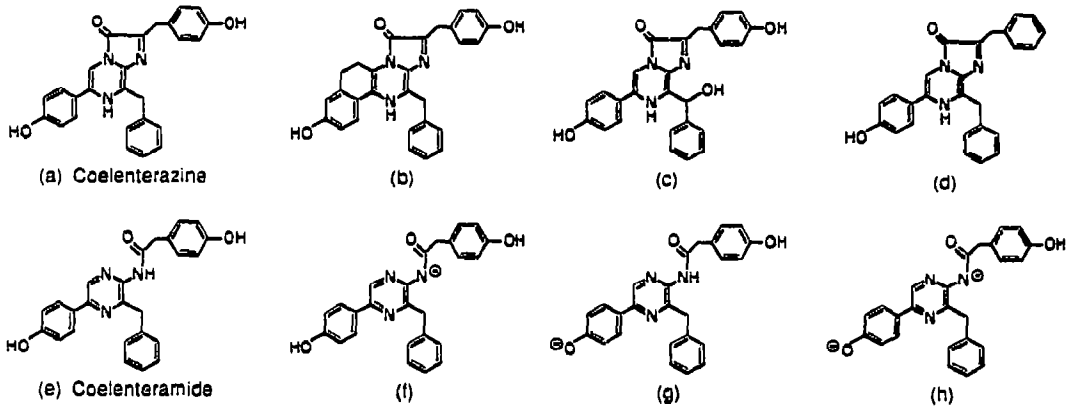


Fig. 1. Chemical structures of coelenterazine analogues and various ionized states of coelenteramide.

small increase in α-helix content. The CD spectrum of apoAQW108F suggests increase in β-sheet content and those of apoAQW129F and apoAQW173F indicate more complex changes, all leading to large losses in activity. Thus, tryptophan modification results in secondary structure changes that are largely associated with losses in bioluminescence activity. However, there is no complete loss of activity and this rules out the possibility that tryptophan may be involved in oxygen binding in the protein.

It is interesting that, with coelenterazine as substrate, AQ emits at 465 nm and AQW86F at 455 nm and 400 nm (Table I and Fig. 2A), whereas with Compound b as substrate, AQ emits at 455 nm and 400 nm, and AQW86F at 400 nm (Table I and Fig. 2B). Such emissions have been noted previously for Compound b with native apoaequorin [18,19]. Thus, depending on whether coelenterazine or Compound b is used, AQ can have a single emission maximum at 465 nm or two maxima at 455 nm and 400 nm. Similarly, AQW86F can have a maximum at 455 nm and a shoulder at 400 nm, or a single maximum at 400 nm. Since the recombinant DNA technique allows for the production of only one molecular species of apoaequorin, apoAQ and apoAQW86F must be identical except for the presence of either a tryptophan or phenylalanine residue at position 86 in the primary structure and a small change in secondary structure for apoAQW86F. The emissions at 455 nm and 400 nm by AQW86F, therefore, must be due to two different excited states of coelenteramide generated by the same apoaequorin molecule and not to the presence of two isoforms of apoaequorin. The tryptophan residue must lie in the active center of the molecule.

A series of studies on the chemiluminescence of coelenterazine and *Vargula* luciferin analogues in hydrophobic media have shown that the emission of coelenteramide at 465 nm is due to a monoanion (Fig. 1, Compound f), that at 400 nm is produced by a neutral molecule (Compound e), and that at 530 nm by a dianion (Compound h) [20–23]. For emission at 465 nm, ~61 kcal/mol (257 kJ · M^{-1}) of energy is required, whereas for the emission at 400 nm, ~71 kcal/mol (299 kJ · M^{-1}) is required, derived from the cleavage of the dioxetanone ring. According to these studies, the bimodal emissions of AQ and AQW86F at 465 nm and 400 nm would be due to two light-emitting species in which first an excited state monoanion (Fig. 1, Compound f) would be

Table I

Bioluminescence activities and emission maxima of regenerated aequorins

Compound		AQ	W12F	W79F	W86F	W108F	W129F	W173F
a.	Luminescence activity[a] (%)	100	13.2	86.0	17.7	3.2	24.6	0.6
	λ_{max}, nm[b]	465	465	460	455	465	465	465
b.	Luminescence activity (%)	100	4.4	124.4	45.6	0.2	1.9	0.3
	λ_{max}, nm	455	400	440	400	N.D.	450	N.D.
c.	Luminescence activity (%)	100	7.6	97.6	9.2	<0.1	6.7	<0.1
	λ_{max}, nm	465	465	465	460	N.D.	465	465
d.	Luminescence activity (%)	100	13.2	63.3	34.3	<0.1	20.6	9.5
	λ_{max}, nm	465	460	455	450	N.D.	465	465

[a] Luminescence activity was calculated as the ratio of the initial maximal light intensity relative to AQ.

[b] Composition of regeneration mixture: 0.1 mg apoaequorin, 10 μg coelenterazine (1 μg/μl, dissolved in absolute methyl alcohol), and 5 μl of 2-mercaptoethanol in 1.0 ml of 30 mM Tris-HCl, pH 7.6/10 mM EDTA. After 6 h of incubation in an ice-bath, a 100-μl aliquot was injected with 1.5 ml of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.6. The spectrum was measured as described in section 2.

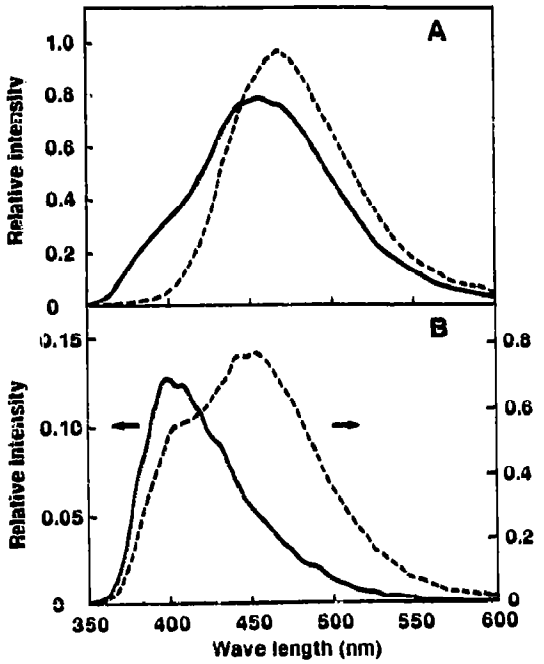


Fig. 2. Emission spectra of aequorin (dashed line) and AQW86F (solid line). (A) The incubation mixture contained 0.1 mg of apoAQ or 0.1 mg of apoAQW86F plus coelenterazine (5 μg) or 0.1 mg of apoAQW86F plus coelenterazine (5 μg) and 2-mercaptoethanol (10 μl) in a total volume of 1.0 ml of 30 mM Tris-HCl, pH 7.6/10 mM EDTA. The mixture was allowed to incubate in an ice bath for 6 h and then a 100-μl aliquot was injected with 1.5 ml of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.6. The spectra were measured as described in section 2. (B) The conditions were the same as in A, except that Compound b (5 μg) was used in place of coelenterazine.

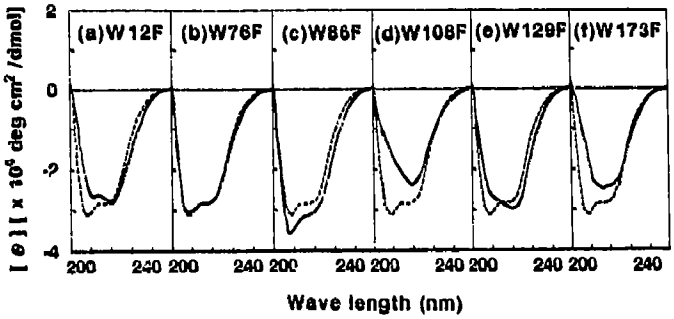


Fig. 3. CD spectra of apoAQ (dashed line) and (a) apoAQW12F, (b) apoAQW79F, (c) apoAQW86F, (d) apoAQW108F, (e) apoAQW129F, and (f) apoAQW173F (solid line). Experimental conditions are described in section 2.

produced, which would then be rapidly protonated to form the excited state neutral molecule (Fig. 1, Compound e). The protonation would occur within the lifetime of the singlet excited state, 10^{-7} to 10^{-9} s, and the neutral molecule would be raised to an energy level of 71 kcal/mol, provided by the binding energy of the proton. This interpretation was supported by a deuterium isotope effect observed when the chemi-luminescence reaction was carried out in D_2O at pH 5.6 [22]. However, we have observed that the *p*-methoxy phenyl (position 6) derivative of coelenterazine was virtually inactive when it was used to regenerate apoAQ and apoAQ86F into aequorin, whereas subsequent addition of coelenterazine to the incubation mixture resulted in full regeneration of AQ and AQW86F activity. Further, Shimomura et al. [18] have reported a similar loss of activity when the same position was occupied by a *p*-amino phenyl group. Thus, a *p*-hydroxyl phenyl group at position 6 in coelenterazine is essential for full bioluminescence activity and it is possible that the excited state of Compound g, with the negatively charged oxygen atom hydrogen bonded to the NH of the indole of tryptophan-86 or bonded to a positively charged nitrogen, is the emitter (465 nm) in the reaction. In our experiments, the fluorescence of coelenteramide was hardly noticeable in Tris-HCl buffer, pH 7.6, but on incubation with apoAQ, a characteristic peak appeared at 460 nm, suggesting that the excited state of Compound g is the emitter in the reaction. Therefore, the excited state emitter at 465 nm is either Compound f or g and the excited state emitter at 400 nm is Compound e (Fig. 1). It is noteworthy that the marine fish, *Porichthys notatus*, also produces a bimodal emission spectrum, with emissions at 485 nm and 507 nm, using as substrate a closely related analogue of coelenterazine, *Vargula* luciferin [24].

Acknowledgements: We are grateful to Dr. Katsunori Teranishi, Mie University, for the gift of Compounds b and c. This work was supported in part by a Grand-in-Aid for Scientific Research in Priority Area (no. 02250102) by the Ministry of Education, Science and Culture of Japan, and by Research Grant DMB-9104684 from the National Science Foundation (F.I.T.).

REFERENCES

[1] Shimomura, O., Johnson, F.H. and Saiga, Y. (1962) J. Cell. Comp. Physiol. 59, 223–239.
[2] Shimomura, O., Johnson, F.H. and Saiga, Y. (1963) J. Cell. Comp. Physiol. 62, 1–8.
[3] Johnson, F.H. and Shimomura, O. (1978) Methods Enzymol. 57, 271–291.
[4] Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T. and Tsuji, F.I. (1985) Proc. Natl. Acad. Sci. USA 82, 3154–3158.
[5] Tsuji, F.I., Inouye, S., Goto, T. and Sakaki, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 8107–8111.
[6] Shimomura, O. and Johnson, F.H. (1973) Tetrahedron Lett. 2963–2966.
[7] Shimomura, O. and Johnson, F.H. (1975) Nature 256, 236–238.
[8] Kurose, K., Inouye, S., Sakaki, Y. and Tsuji, F.I. (1989) Proc. Natl. Acad. Sci. USA 86, 80–84.
[9] Caruthers, M.H. (1987) in: Synthesis and Applications of DNA and RNA (S.A. Narang, Ed.), Academic Press, Orlando, Florida, pp. 47–94.
[10] Inoue, S., Sugiura, S., Kakoi, H., Hasizume, K., Goto, T. and Iio, H. (1975) Chem. Lett. 141–144.
[11] Inouye, S., Aoyama, S., Miyata, T., Tsuji, F.I. and Sakaki, Y. (1989) J. Biochem. 105, 473–477.

[12] Higuchi, R., Krummel, S. and Saiki, R.K. (1988) Nucleic Acids Res. 16, 7351–7367.
[13] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 51–59.
[14] Hattori, M. and Sakaki, Y. (1986) Anal. Biochem. 152, 232–238.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning, A laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[16] Hastings, J.W. and Weber, G. (1965) Photochem. Photobiol. 4, 1049–1050.
[17] Shimomura, O. and Shimomura, A. (1981) Biochem. J. 199, 825–828.
[18] Shimomura, O., Musicki, B. and Kishi, Y. (1988) Biochem. J. 251, 405–410.
[19] Shimomura, O., Musicki, B. and Kishi, Y. (1989) Biochem. J. 261, 913–920.
[20] McCapra, F. and Chang, Y.C. (1967) J. Chem. Soc. Chem. Commun. 1011–1012.
[21] Goto, T., Inoue, S. and Sugiura, S. (1968) Tetrahedron Lett. 3873–3876.
[22] Goto, T., Inoue, S., Sugiura, S., Nishikawa, K., Isobe, M. and Abe, Y. (1968) Tetrahedron Lett. 4035–4038.
[23] Hori, K., Wampler, J.E. and Cormier, M.J. (1973) J. Chem. Soc., Chem. Commun. 492–493.
[24] Tsuji, F.I., Nafpaktitis, B.G., Goto, T., Cormier, M.J., Wampler, J.E. and Anderson, J.M. (1975) Mol. Cell. Biochem. 9, 3–8.