

Mass spectrometric evidence for a disulfide bond in aequorin regeneration

Yoshihiro Ohmiya^a, Sadamu Kurono^b, Mamoru Ohashi^b, Thomas F. Fagan^c, Frederick I. Tsuji^{c,d,*}

^a*PRESTO, JRDC, Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan*

^b*Department of Applied Physics and Chemistry, The University of Electro-Communications, Chofu, Tokyo 182, Japan*

^c*Osaka Bioscience Institute, 6-2-4 Furuedai, Osaka 565, Japan*

^d*Marine Biology Research Division 0202, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA*

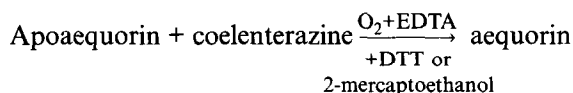
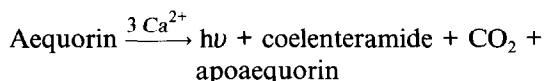
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Tryptic digests of purified recombinant apoaquorin were analyzed, before and after reduction with DTT, by fast atom bombardment mass spectrometry. The results showed that apoaquorin contains a disulfide bond between Cys¹⁴⁵ and Cys¹⁵² and that the reduction of this bond is involved in the regeneration of aequorin.

Bioluminescence; Photoprotein; Ca²⁺-binding protein; Coelenterazine

1. INTRODUCTION

Aequorin is a small Ca²⁺-binding photoprotein found in the jellyfish, *Aequorea victoria*, which emits light by an intramolecular reaction when triggered by Ca²⁺ [1]. Aequorin consists of apoaquorin (a monomeric apoprotein, m.wt. = 21,400), molecular oxygen and coelenterazine (an imidazopyrazine compound, m.wt. = 423). Apoaquorin consists of 189 amino acid residues with three EF-hand structures (Ca²⁺-binding sites) [2,3]. On binding Ca²⁺, aequorin is converted to an enzyme (luciferase), which catalyzes the oxidation of coelenterazine by the bound oxygen, yielding as products light ($\lambda_{\text{max}} = 470$ nm), CO₂ and coelenteramide (the oxidation product of coelenterazine). Apoaquorin may be regenerated into aequorin by incubation with coelenterazine, molecular oxygen, EDTA and 2-mercaptoethanol [4] or dithiothreitol (DTT) [5].



In the regeneration of apoaquorin, EDTA is used to chelate Ca²⁺, but the role of DTT or 2-mercaptoethanol is not clearly understood. Since active aequorin is ob-

tained only when DTT or 2-mercaptoethanol is added to the incubation mixture [4,5], it is presumed that the reducing agent serves to reduce a disulfide bond. The aequorin molecule contains Cys¹⁴⁵, Cys¹⁵² and Cys¹⁸⁰ [2,3], but it is not known whether these residues form a disulfide bond. Site-directed mutagenesis studies have been carried out in which cysteine has been replaced with serine, but the results have been inconclusive [5,6]. Five of the seven mutant aequorins which had their cysteine residues replaced singly or doubly with serine had reduced bioluminescence activity, whereas the sixth with C¹⁴⁵ and C¹⁵² replaced with serine had almost no activity, while the seventh with all three cysteine residues substituted with serine had slightly higher activity than the wild type control. From these results, it was not possible to conclude whether a disulfide bond is formed between two of the three cysteine residues. The present paper presents mass spectrometric evidence that the apoaquorin molecule contains a disulfide bond between Cys¹⁴⁵ and Cys¹⁵² and that in the apoaquorin regeneration reaction cleavage of this bond occurs.

2. MATERIALS AND METHODS

2.1. Materials

Immobilized trypsin (bovine pancreas, TPCK treated) was obtained from Pierce (Rockford, IL), dithiothreitol (DTT) from Wako Pure Chemicals (Osaka, Japan), *N*-ethylmorpholine from Sigma Chemical Co. (St. Louis, MO) and 1-thioglycerol from Tokyo Kasei (Tokyo). Coelenterazine was chemically synthesized [7]. 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 pIP-HE containing the apoaquorin gene fused to the ompA secretion peptide

*Corresponding author. Fax: (1) (619) 534 7313.

Table I

Predicted and observed masses of peptides in tryptic digest of apo-aequorin before and after reduction with DTT

Peptide fragment	Predicted MH ⁺	Observed (m/z)	
		Before reduction	After reduction
(3–11)	1,064	1,064.8	1,064.8
(12–15)	531	531.5	N.D.
(16–17)	301	N.D.	N.D.
(18–30)	1,573	1,574.1	1,574.1
(31–39)	1,097	1,098.0	1,097.6
(40–56)	1,741	1,741.7	1,741.2
(57)	174	N.D.	N.D.
(58–59)	301	N.D.	N.D.
(60–72)	1,300	1,300.3	N.D.
(73–87)	1,814	1,814.5	1,814.2
(88)	146	N.D.	146.3
(89–96)	918	918.8	918.4
(97–99)	380	N.D.	N.D.
(100–106)	842	842.7	842.5
(107–118)	1,392	1,392.1	1,392.0
(119–130)	1,390	1,391.3	1,391.1
(131–134)	482	N.D.	N.D.
(135–150)	1,756	N.D.	1,756.3
(151–167)	1,925	N.D.	1,925.2
(168–182)	1,826	N.D.	N.D.
(183–189)	676	676.4	676.5
(135–150)ss(151–167)	3,681	N.D.	N.D.
(135–150)ss(168–182)	3,582	N.D.	N.D.
(151–167)ss(168–182)	3,751	N.D.	N.D.

N.D., not detected.

wild type aequorin [5]. These results suggest that in the regeneration of aequorin, the thiol group of cysteine, and the serine hydroxyl in the case of the serine mutant, plays a role in the incorporation of coelenterazine into the apoprotein.

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