Volume 332, number 3, 226–228 FEBS 13111 October 1993

Mass spectrometric evidence for a disulfide bond in aequorin regeneration

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Received 20 August 1993

Tryptic digests of purified recombinant apoaequorin were analyzed, before and after reduction with DTT, by fast atom bombardment mass spectrometry. The results showed that apoaequorin contains a disulfide bond between Cys^{145} and Cys^{152} and that the reduction of this bond is involved in the regeneration of aequorin.

Bioluminescence; Photoprotein; Ca^{2+}-binding protein; Coelenterazine

1. INTRODUCTION

Aequorin is a small Ca^{2+}-binding photoprotein found in the jellyfish, *Aequorea victoria*, which emits light by an intramolecular reaction when triggered by Ca^{2+} [1]. Aequorin consists of apoaequorin (a monomeric apoprotein, m.wt. = 21,400), molecular oxygen and coelenterazine (an imidazopyrazine compound, m.wt. = 423). Apoaequorin consists of 189 amino acid residues with three EF-hand structures (Ca^{2+}-binding sites) [2,3]. On binding Ca^{2+}, aequorin is converted to an enzyme (luciferase), which catalyzes the oxidation of coelenterazine by the bound oxygen, yielding as products light (λ_{max} = 470 nm), CO_2 and coelenteramide (the oxidation product of coelenterazine). Apoaequorin may be regenerated into aequorin by incubation with coelenterazine, molecular oxygen, EDTA and 2-mercaptoethanol [4] or dithiothreitol (DTT) [5].

$$\text{Aequorin} \xrightarrow{3\,Ca^{2+}} h\upsilon + \text{coelenteramide} + CO_2 + \text{apoaequorin}$$

$$\text{Apoaequorin} + \text{coelenterazine} \xrightarrow[+\text{DTT or 2-mercaptoethanol}]{O_2+\text{EDTA}} \text{aequorin}$$

In the regeneration of apoaequorin, EDTA is used to chelate Ca^{2+}, but the role of DTT or 2-mercaptoethanol is not clearly understood. Since active aequorin is obtained 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 Cys^{145}, Cys^{152} and Cys^{180} [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 C^{145} and C^{152} 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 apoaequorin molecule contains a disulfide bond between Cys^{145} and Cys^{152} and that in the apoaequorin 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 apoaequorin gene fused to the ompA secretion peptide

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coding sequence under the control of the *lpp* promoter and *lac* operator, which allowed apoaequorin to be secreted directly into the culture medium [8].

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

The transformed *E. coli* D1210 was cultured as previously described [9], except that LB medium was used throughout. The apoaequorin was precipitated from the culture medium by acidification [8] and purified by anion exchange chromatography using a Mono Q (Pharmacia) column with a linear gradient of 0.1 to 0.6 M NaCl in 30 mM Tris-HCl, pH 7.6, and by gel filtration using a Superose 12 (Pharmacia) column with 30 mM Tris-HCl, pH 7.6, as eluting buffer. The purified apoaequorin gave a single band on SDS-PAGE (12.5%) with an estimated purity of >95% and was fully active when regenerated with coelenterazine [9].

2.4. *Digestion of apoaequorin with immobilized trypsin*

The purified apoaequorin was dialyzed against two changes of 100 mM NH_4HCO_3, pH 8.0, and the immobilized trypsin was washed three times with 500 μl each of 100 mM NH_4HCO_3, pH 8.0. Digestion was carried out by mixing 1.0 mg of apoaequorin/ml of 100 mM NH_4HCO_3, pH 8.0, with 0.25 ml of the immobilized trypsin and incubating the mixture with shaking for 24 h at 38°C. After centrifuging to remove the immobilized trypsin, the supernatant containing the digest was dried under vacuum.

2.5. *Analysis of digest by mass spectrometry*

The dried digest was analyzed by fast atom bombardment (FAB) mass spectrometry [10] using a Finnigan MAT TSQ-700 mass spectrometer. The sample was dissolved in 10% acetic acid to give a concentration of approximately 200 pmol of peptide/μl; 1.0 μl of the solution was then mixed with 0.6 μl of glycerol (matrix) and applied to the copper probe. After completion of a mass spectrometric determination, 1.0 μl of a 1:1 (v/v) mixture of *N*-ethylmorpholine, pH 8.5, and 1 M DTT was applied to the same probe and allowed to stand for 10 min at room temperature. After drying, a mixture of 0.5 μl of 10% oxalic acid and 0.7 μl of 1-thioglycerol was applied to the probe and the analysis repeated. The mass scale was calibrated with cesium iodide. The FAB probe was operated at 8.0 kV and the spectrometer was set at a resolution of 2000. All spectra were recorded in the positive mode.

3. RESULTS AND DISCUSSION

Previous SDS-PAGE and Western blot studies have indicated that recombinant apoaequorin contains more than one molecular species, possibly due to the formation of intra-disulfide bonds [11,12]. In the present study, SDS-PAGE analysis of purified apoaequorin reveale the presence of two distinct bands, one at 21.5 kDa under completely oxidizing conditions and another at 25.0 kDa under completely reducing conditions; under partially oxidizing conditions both the 21.5 kDa and 25.0 kDa bands were observed (data not shown). Since the calculated molecular size for recombinant apoaequorin is 21.5 kDa, it is apparent that the 25.0 kDa value under reducing conditions is due to an increase in hydrodynamic volume and that only one molecular species exists. Apoaequorin has three cysteine residues, C^{145}, C^{152} and Cys^{180} (Fig. 1) which allows for a single disulfide bond to be formed, leaving the third free. With such a disulfide bond in apoaequorin, digestion of the protein under non-reducing conditions with

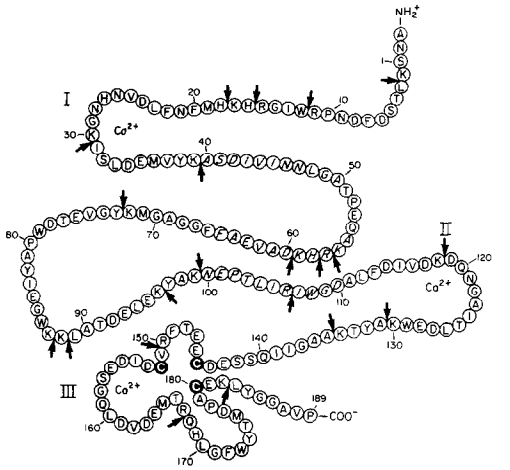

Fig. 1. Primary structure of secreted apoaequorin showing the trypsin cleavage sites (arrows). The N-terminal amino acid residue in the mature native apoaequorin is presumed to be valine [6], which is replaced in secreted apoaequorin by serine plus asparagine and alanine due to the use of the ompA secretion signal, which is removed on export of the apoaequorin [8,11]. The numbering of the amino acid residues and the three Ca^{2+}-binding sites is the same as shown in a previous schematic diagram for native apoaequorin [6].

trypsin should yield 19 peptide fragments, with the bond contained in one of the following fragments: (135–150)$C^{145}C^{152}$(151–167), (135–150)$C^{145}C^{180}$(168–182) and (151–167)$C^{152}C^{180}$(168–182). Subsequent reduction of the fragment should yield two smaller fragments detectable by mass spectrometry.

The results of a mass spectral analysis of a tryptic digest of apoaequorin are presented in Table I. The table lists the predicted masses and the observed masses of the tryptic fragments before and after reduction. Two unambiguous peaks were observed in the mass spectrum at *m/z* 1756.3 and 1925.6, corresponding to the fragments (135–150) and (151–167), respectively. The observance of these two peaks is indicative of a disulfide bond between Cys^{145} and Cys^{152}. No other potential fragment was observed. The 1826 mass peak of fragment (168–182) was inexplicably absent from the mass spectrum; possibly the reactivity of the free thiol group of Cys^{180} caused the fragment to be bound by another group. Thus, on formation of the Cys^{145}–Cys^{152} bond, an internal peptide loop is formed which blocks the incorporation of coelenterazine, and during regeneration of apoaequorin into aequorin, this bond is cleaved by the added DTT or 2-mercaptoethanol.

The regeneration of apoaequorin into aequorin, however, does not depend solely on the reduction of the Cys^{145}–Cys^{152} bond since: (i) substitution of Cys^{145} and Cys^{152} with serine leads to almost complete loss of activity, whereas the replacement of all three cysteine residues with serine results in a fully active mutant aequorin, and (ii) the regeneration time of the serine-substituted apoaequorin is about three times longer than

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.

Acknowledgements: Thomas F. Fagan was supported by a post-doctoral fellowship from the Science and Technology Agency of Japan. This research was supported in part by Research Grant MCB91-04684 from the National Science Foundation (F.I.T.).

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