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Selective protein modification by the hydroperoxide intermediate in a photoprotein, aequorin

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ABSTRACT

During the course of protein modification program, we employed a recombinant aequorin, the apo-protein reconstituted with coelenterazine, and found out that the photolytic hyperperoxide modified three –S–$SCH_2CHOHCHOHCH_2SH$ groups to –S–$SCH_2CHOHCH{=}CH{-}S{=}O)H$ or –S–$SCH_2CHOHCH{=}CH{-}S(=O)OH$ of terminal DTT connected to cysteine residues of the C^{145}, C^{152} and C^{180}, which turned out to locate near the chromophore.

1. Introduction

Exposure of proteins to the reactive oxygen species (ROS) such as hydroxyl radical causes gross structural modification.[1] We have proven that pin-point oxidation by hydroxyl radical took place selectively on the amino acid residues such as histidine, leucine, proline, which were located within limited distance (ca. 7 Å) from Cu atom of the Zn,Cu-SOD (super-oxide dismutase).[2] In order to generate the ROS, we pointed out photo irradiation of hydroperoxide. In 1976, Ogata reported that the α-hydroperoxy ketones generates the ROS by photo irradiation.[3] The photolysis of hydrogen peroxide provides some ROS and the resulting ROS must selectively oxidize some amino acid residues. Therefore we have planned photooxidative modification of photoprotein, aequorin, containing hydroperoxide as a substrate.

Aequorin is a well known calcium-sensitive photoprotein, found in a luminous jellyfish *Aequorea aequorea*.[4] It contains coelenterazine (8-benzyl-2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl) imidazo[1,2*a*]pyrazine-3(7*H*)-one, **1**, Fig. 1) in the form of (2*S*)-hydroperoxide (chromophore (**2**), Fig. 1) as a chromophore reported by X-ray crystallography studies by Head et al. (Fig. 2).[5] Apoaequorin consists of 189 amino acid residues with four EF-hand domains; three of them can bind calcium ions. Reconstitution

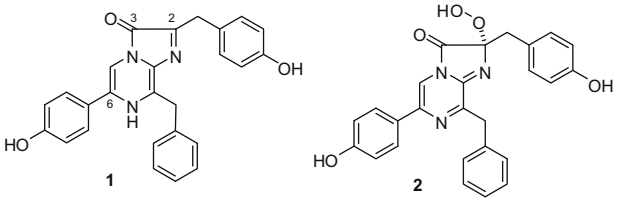

Figure 1. Coelenterazine **1** and the chromophore **2** in a photoprotein, aequorin.

of apoaequorin with coelenterazine affords active aequorin by incubation under air atmosphere with a buffer containing EDTA, 2-mercaptoethanol (2ME) or dithiothreitol (DTT).[6] In the absence of 2ME or DTT, aequorin gradually loses its activity down to 30% compared with the original luminescence. At pH 7.5 calcium ions work as a trigger for emitting blue light (470 nm).

In 1996, we have reported photochemical oxidation using doubly ^{13}C labeled coelenterazine analog **3** to yield the peroxide intermediates **4**: (2*S*)-hydroperoxide as well as **5**, a dioxetanone; peroxy-β-lactone as shown in Figure 3.[7] These two compounds were dissolved and photolyzed in a mixture of CF_3CD_2OD and CD_3OD (7:3) solvents at −78 °C with bubbling oxygen and analyzed by low-temperature NMR. The half life time of **4** was ca. 2 min at 0 °C in this solvent. Solvent effect in this low temperature photooxidation was critically important to give these peroxidic products, which further decomposed by elongating photolysis

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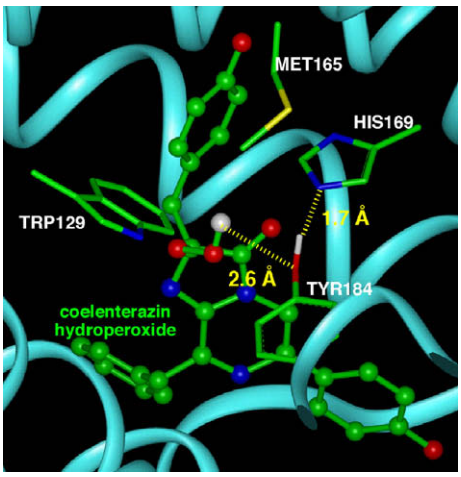

Figure 2. Conformation of **2** in an aequorin analyzed by using X-ray crystallography.

time.[7] However, it is noteworthy that similar hydroperoxide is kept stable in aequorin due to sharing the peroxidic proton with tyrosine residue in this protein. By using this stability we have embarked upon photooxidative modification of aequorin with the hydroperoxide **2**, and implemented photo irradiation. In the resultant photolyzed solutions, we found oxidation products in both of aequorin solution with **1**. It was interesting to learn what kind of protein modification occurred as the results through subsequent analyses after tryptic digestion into peptides. We became interested in tracing the details of structural modification because of the following reasons as: (1) this is the only luminescent intermediate, which has a long lifetime, in nature, (2) following the fate of this hydroperoxide without luminescence, and (3) a case study of high yield protein modification.

2. Results and discussion

2.1. Modified amino acid analysis by photo irradiated aequorin by means of LC–MS

Aequorin was reconstituted from apoaequorin by incubation with **1** in Tris buffer (pH 7.5) for 2 h at 0 °C,[8] and then the resulting aequorin was subjected to photolysis by using a high-pressure mercury lamp (365 nm) for 20 min at room temperature under air. Sample was digested with trypsin in Tris buffer (pH 7.5) for 12 h at 37 °C. The resulting tryptic peptides were then analyzed by LC–MS.

Apoaequorin has three cysteine residues; thus, C^{145}, C^{152} and C^{180}; where C^{145} and C^{152} forms a disulfide bond and C^{180} exists as free SH form.[9] Under DTT-containing condition where the disulfide bond cleaves, trypsin digests of aequorin yielded all the 23 peptide fragments except for T19 in the mass spectra. When LC–MS data were analyzed with tryptic peptides by comparing with and without photo irradiation, we found molecular weight changes in the three peptides (T20, T21 and T22) out of 23 peptides. Without photo irradiation these three peptides appeared as T20 886.4 u, T21 962.9 u, and T22 904.9 u as all data being +2 charged ions. With photo irradiation all these three peptides appeared as T20 961.5 u and 969.5 u; T21 1037.9 u and 1045.9 u; T22 979.9 u and 987.9 u, as shown in Figure 4 by LC–MS/MS of ESI-ion-trap. The respective molecular weights increased in 150 or 166 u, which were assigned to a dehydrated mono/di oxidized DTT. In the chromatogram of T21 after irradiation, a noticeable peak (962.9 u) was detected, but it was proved to be an isotope peak of modified T20 (961.5 u). We also detected two kinds of peaks in the Figure 4 chromatograms of modified T21 (1045.9 u) and modified T22 (987.9 u), respectively. Left peak identified as an adduct of mono-oxidized DTT on cysteine (150 u) and oxidized methionine (16 u), while right peak did as an adduct of di-oxidized DTT on cysteine (166 u) of their peptides.

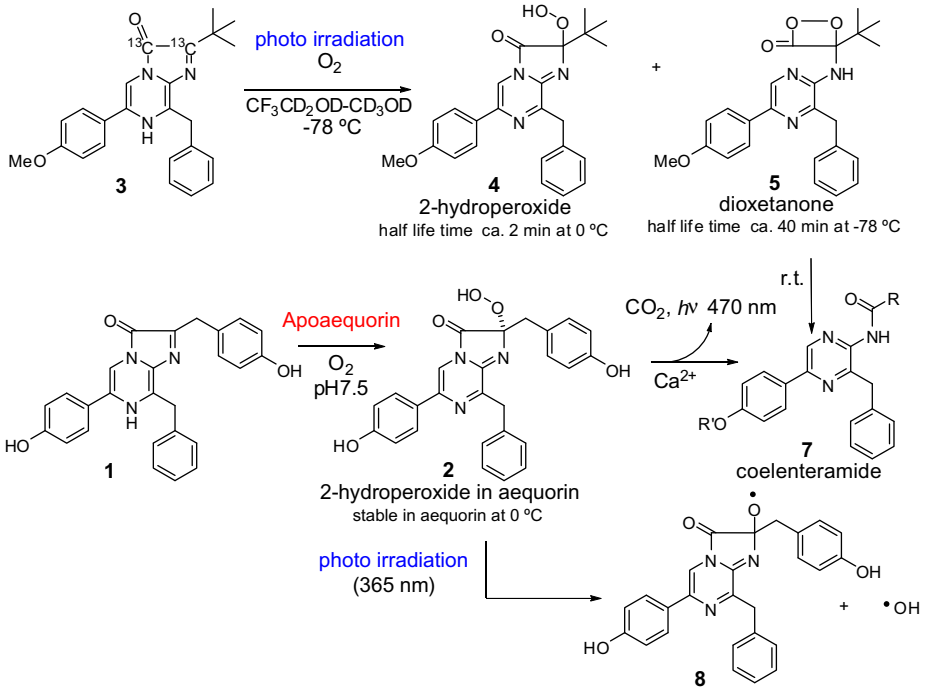

Figure 3. A chemiluminescent intermediate **4** as a photoproduct and a bioluminescent intermediate **2** in aequorin. Photolysis of **2** in aequorin gave active oxygen species **7** and hydroxyl radical.

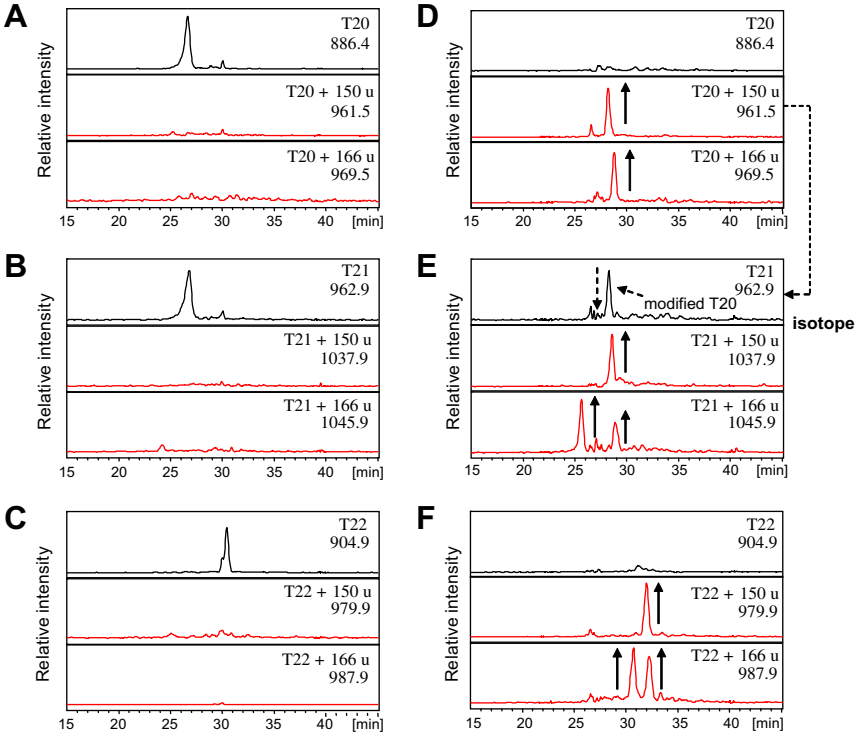

Figure 4. Ion chromatogram of T20 and T21, T22 peptides before and after photo irradiation, respectively, eluted with the PPG system. (A) Ion chromatogram of T20 and modified T20 peptides before photo irradiation. (B) Ion chromatogram of T21 and modified T21 peptides before photo irradiation. (C) Ion chromatogram of T22 and modified T22 peptides before photo irradiation. (D) Ion chromatogram of T20 and modified T20 peptides after photo irradiation. (E) Ion chromatogram of T21 and modified T21 peptides after photo irradiation. (F) Ion chromatogram of T22 and modified T22 peptides after photo irradiation (middle figure: DTT adduct of cysteine residue was converted into mono-oxidized DTT (+150). below figure: DTT adduct of cysteine residue was converted into mono-oxidized DTT (+150) and methionine residue was oxidized to give sulfoxide (+16) or DTT adduct of cysteine residue was converted into di-oxidized DTT (+166), respectively.)

The results of mass spectrometric analysis are summarized in Table 1. The predicted mass numbers and the observed mass numbers of the digests are shown concerning to the effect of photo irradiation time. The modified points were proved to be selective on the three cysteine residues. As an example, two spectra of T20 with (A) and without (B) photo irradiation are shown in Figure 5. It is clear that C^{145} have selectively modified; and the ion peak was assigned as adducts of oxidized DTT with cysteine. We found the same results with C^{152} in T21 and C^{180} in T22. Some methionine residues in T21 and T22 were also oxidized only after the photo irradiation.

The photolysis of **2** might provide some ROS as **7** and the resulting ROS must selectively oxidize sulfur atoms in aequorin. This is contrastive to our previous results[2]; we reported that hydroxyl radical, which was produced by the reaction of Zn, Cu-SOD with H_2O_2, selectively oxidized histidine, leucine, and proline, which were located within limited distance (ca. 7 Å) from Cu atom of the Zn, Cu-SOD.

We have applied this selective oxidation by ROS obtained from the photolysis of hydroperoxide (**2**). In this oxidation reaction, only terminal sulfur atoms of DTT bound to cysteine were oxidized. Partial oxidations were also occurred at methionine residues, which were assumed to be oxidized to sulfoxide. On the other hand, oxidation of H^{169}, which locates at the distance of 3.75 Å from coelenterazine hydroperoxide, did not occur at all during this photo irradiation.

2.2. Effects of photo irradiation to the luminescence ability and to oxidative modification

During the analysis of LC–MS data obtained for tryptic peptides comparing photolyzed apoaequorin with photolyzed aequorin, we

Table 1
Predicted and observed mass numbers of trypsin-digested peptides containing cysteine residues of aequorin and apoaequorin before and after photo irradiation

	Peptide fragment	Predicted mass $[M+2H]^{2+}$	Observed (m/z)			
			Before irradiation		After irradiation	
			Aequorin	Apoaequorin	Aequorin	(Detected)
T20	SAGIIQSSEDC[145]EETFR	886.4	886.4	886.4	N.D.	961.5 969.5
T21	VC[152]DIDESGQLDVDEMTR	962.9	962.9	962.9	N.D.	1037.9 1045.9
T22	QHLGFWYTMDPAC[180]EK	913.4	**904.9**[a]	**904.9**[a]	N.D.	979.9 987.9

N.D.; not detected.
[a] See note.[13]

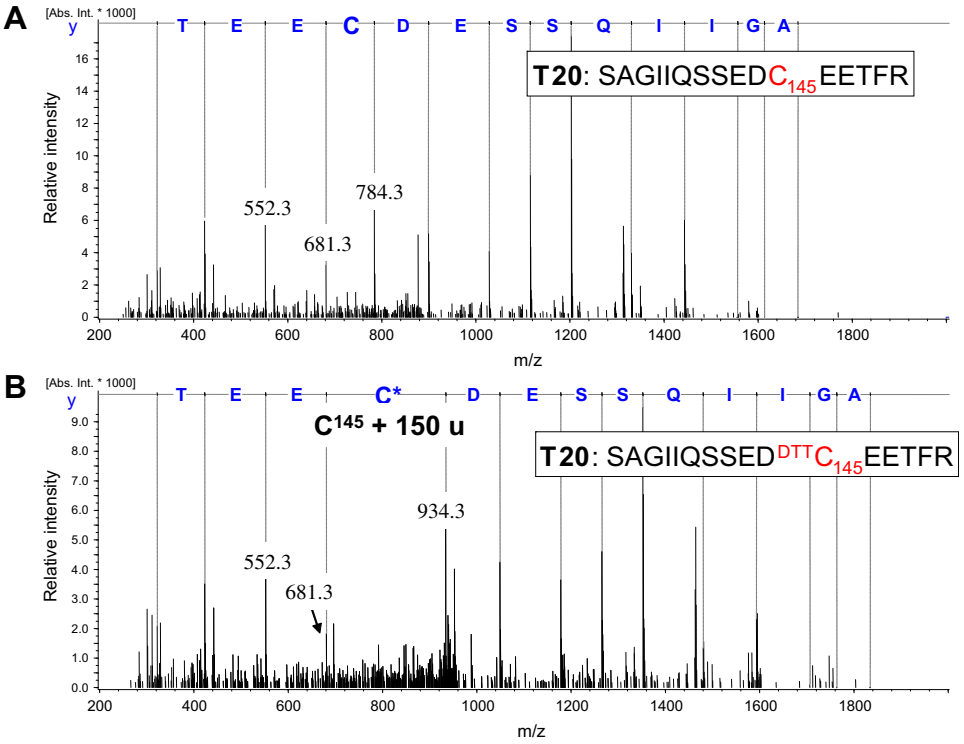

Figure 5. Tandem mass spectra of tryptic peptide T20 before (A) and after (B) photo-irradiation of aequorin reducted with DTT.

noticed the molecular ion peaks of T20, T21 and T22 peptides only changed in photolyzed aequorin but not in apoaequorin. The chromophore is indispensable for the oxidation.

In order to confirm the fact, we measured each luminescent activity of aequorin by addition of Ca^{2+}, after photolysis every minutes. Because we thought the chromophore **2** should be consumed by photolysis in order to produce ROS, therefore the luminescent activity of aequorin should be decreased along with duration of photolysis. Actually, the luminescent activity gradually decreased in accordance with photo irradiation time, and it completely disappeared in 7 min (Fig. 6, bar graph). Furthermore, we performed LC–MS analysis of trypsin digest of aequorin, which were irradiated for 5, 10, 15 and 20 min, by using a nano-HPLC-ESI-IT-MS, -MS/MS and PPG solvent solution program. As shown in Figure 6, T20, T21, and T20 were decreased in accordance with photolysis and totally disappeared in 15 min, and on the other hand, modified T20+**11** (+**12**), T21+**11** (+**12**), and T22+**11** (+**12**) were increased in time dependent manner of photolysis by reacting with ROS.

Thus, we confirmed that the oxidation of these T20–T22, in which contained cysteine residues, were dependent for the consumption of chromophore **2** by photolysis.

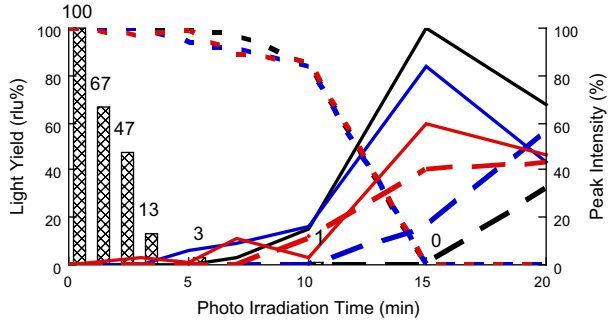

Figure 6. Bar graph: The bioluminescent activity (rlu%) of aequorin depending on photo irradiation time. Line graph: The ratio of three modified peptide fragments T20, T21 and T22 based on mass peak intensity. T20 (black dotted line) and T21 (blue dotted line) and T22 (red dotted line), T20 + **11** (black dashed line) and T21 + **11** (blue dashed line) and T22 + **11** (red dashed line), T20 + **12** (black line) and T21 + **12** (blue line) and T22 + **12** (red line). **11**: sulfenic acid, **12**: sulfinic acid.

2.3. Photooxidative modifications of methionine residues

Some methionine residues were also found to be oxidized and these modifications of methionine seemed to be related to the photo irradiation. We compared the rate of oxidation on methionine before and after photo irradiation for 20 min (Fig. 7). Aequorin has five methionine residues M^{19}, M^{36}, M^{71}, M^{165} and M^{176} which were partially oxidized to form sulfoxide before the photo irradiation. The amount of the oxidation increased after irradiation, and it especially modified M^{165} and M^{176} obtained from only irradiated aequorin. Although these modifications seemed to be unrelated to the distances between methionine residues and the chromophore **2**, we thus concluded that these modifications were derived from photo irradiated chromophore.

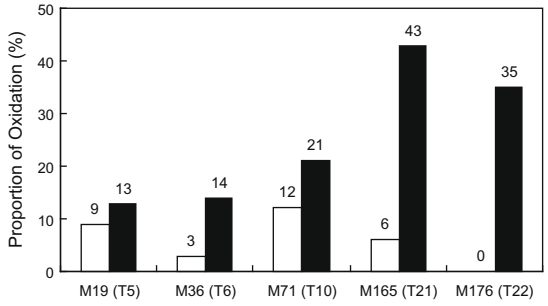

Figure 7. The ratio of photooxidation on methionine residues before (white) and after (black) photo irradiation based on mass peak intensity.

Table 2
Predicted and observed masses of peptides in trypsin proteolytic digests of apoaequorin and aequorin before and after photo irradiation reduced with 2ME (78 u) and DTT (154 u)

	Peptide fragment	Predicted mass $[M+2H]^{2+}$	Observed									
			Before irradiation Aequorin		After irradiation Apoaequorin				After irradiation Aequorin			
			2ME	DTT	2ME	Detected	DTT	Detected	2ME	Detected	DTT	Detected
T1–T2	HKLTPDFDNPK	656.3	656.3	656.3	656.3		656.3		656.3		656.3	
T2–T3	LTPDFDNPKWIGR	779.9	779.9	779.9	779.9		779.9		779.9		779.9	
T4–T5	HKHMFNFLDVNHNGR	933.5	933.5	933.5	933.5		933.5		933.5		933.5	
T6	ISLDEMVYK	549.3	549.3	549.3	549.3		549.3		549.3		549.3	
T7–T8	ASDIVINNLGATPEQAKR	949.0	949.0	949.0	949.0		949.0		949.0		949.0	
T9–T10	HKDAVEAFFGGAGMK	782.9	782.9	782.9	782.9		782.9		782.9		782.9	
T11–T12	YGVETEWPEYIEGWKR	650.3	650.3	650.3	650.3		650.3		650.3		650.3	
T13–T14	LASEELKR	473.3	473.3	473.3	473.3		473.3		473.3		473.3	
T15–T16	YSKNQITLIR	618.4	618.4	618.4	618.4		618.4		618.4		618.4	
T17	LWGDALFDIIDK	703.4	703.4	703.4	703.4		703.4		703.4		703.4	
T18	DQNGAISLDEWK	688.3	688.3	688.3	688.3		688.3		688.3		688.3	
T19	AYT K	482.3[a]	N.D.	N.D.	N.D.		N.D.		N.D.		N.D.	
T20	SAGIIQSSEDCEETFR	886.4	886.4	886.4	886.4	**924.5**	886.4	**N.D.**	N.D.	**924.5**	N.D.	**961.5** **969.5**
T21	VCDIDESGQLDVDEMTR	962.9	962.9	962.9	962.9	**1000.9** **1008.9**	962.9	**N.D.**	N.D.	**1000.9** **1008.9**	N.D.	**1037.9** **1045.9**
T22	QHLGFWYTMDPACEK	913.4	**904.9**[c]	**904.9**[c]	**904.9**[c]	**942.9** **950.9**	**904.9**[c]	**N.D.**	N.D.	**942.9** **950.9**	N.D.	**979.9** **987.9**
T23	LYGGAVP	676.4[a]	676.4[a]	676.4[a]	676.4[a]		676.4[a]		676.4[a]		676.4[a]	
T20–T21	SAGIIQSSEDCEETFR / VCDIDESGQLDVDEMTR	1226.5[b]	1226.4[b]	1226.4[b]	1226.4[b]		1226.4[b]		1226.4[b]		1226.4[b]	

2.4. Confirmation of adduct on cysteine residues by using 2ME

In order to confirm the structure of adduct on cysteine residues, we used 2ME instead of DTT for reconstitution of aequorin. Under 2ME containing condition where the disulfide bond cleaves, digestion of aequorin with trypsin also afforded all the 23 peptide fragments except for T19 as separated peaks in LC–MS. We found different changes of the molecular weight took place in the three peptides (T20, T21 and T22) out of 23 peptides; thus, T20 886.4 u to 924.5 u, T21 962.9 u to 1000.9 and 1008.9 u, and T22 904.9 u to 942.9 and 950.9 u; all data being +2 charged ions).

We noticed the molecular ion peaks of T20, T21 and T22 peptides completely disappeared in mass spectra after photo irradiation (Table 2). The respective molecular weights increased in 76 and/or 92 u, which were assigned as adducts of 2ME with cysteines and oxidized methionines from molecular weight. However, these adducts of 2ME were not oxidized at all.

We considered that ROS generated from the chromophore selectively oxidized sulfhydryl group. Although sulfhydryl group of DTT added to cysteine could cleave the own disulfide bond between cysteine and DTT, photooxidized sulfhydryl group of DTT on cysteine lost the ability to cleave them. These results lead us to conclude that only aequorin after photo irradiation could yield the photooxidative modifications.

2.5. Consideration of the photooxidation mechanism of aequorin

We supposed that sulfhydryl groups of DTT attached to these cysteine residues were selectively oxidized and then dehydrated to give both sulfenic acid (**11**; + 150 u) and sulfinic acid (**12**; + 166 u) as outlined in Figure 8. On the basis of relative MS peak intensities, we calculated the ratio of **11** and **12** to be 1:1.5–5, respectively (Fig. 6). The distance between sulfhydryl groups of these cysteine residues and the hydroxyl group of peroxide was estimated to be shorter than 13 Å by using a MacroModel (OPLS_2001, PDB file of aequorin: 1EJ3).[10] Such modification was never found in apoaequorin being photo irradiated in the same way, because it does not absorb at 365 nm. It happened specifically to the bound substrate hydroperoxide (**2**) in the aequorin that is the origin of the photolytic reaction. These results lead us to conclude that photolysis of reconstituted aequorin generated a reactive oxidant, which converted DTT-cysteine residues locating nearby the peroxide into **11** and **12** derivatives.

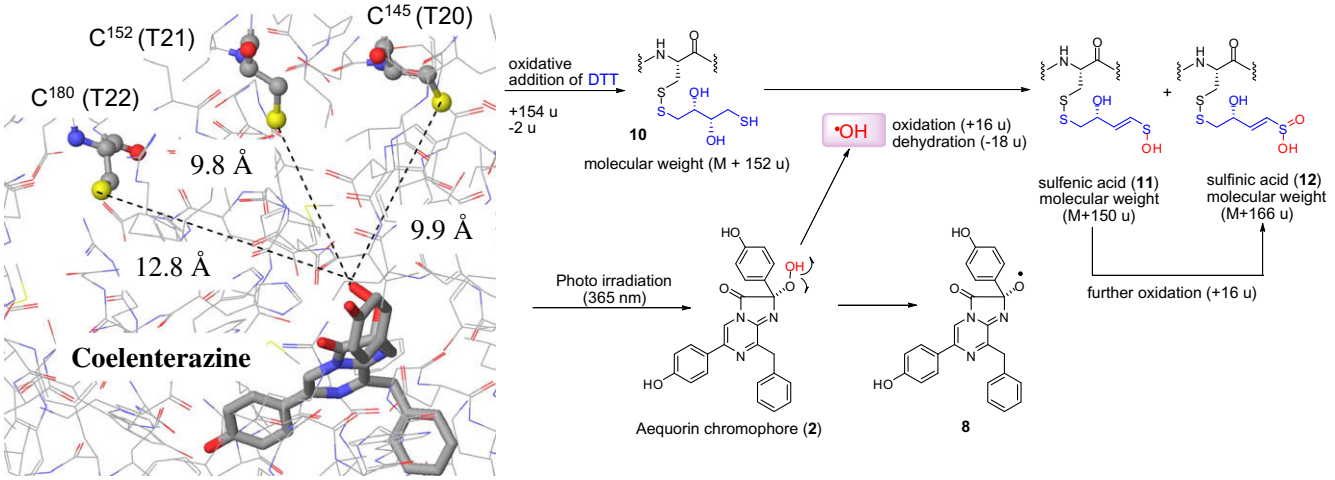

Figure 8. The mechanism of oxidative modification of DTT-cysteine residues caused by ROS such as hydroxy radical generated from photolysis of coelenterazine hydroperoxide. The distance between sulfhydryl groups of these cysteine residues and the hydroxyl group of peroxide was estimated to be shorter than 13 Å by using a MacroModel (OPLS_2001, PDB file of aequorin: 1EJ3).[10]

3. Conclusions

As conclusion, the photo oxidative modifications described herein are the first report of its kind of photoprotein. This paper also describes the first example where luminescent intermediate works as a new device for labeling around the active site of aequorin. This method would be applicable in order to study other photoproteins whether they form a stable hydroperoxide intermediate as a bioluminescent intermediate through the selective oxidation and analysis by means of LC-mass spectrometry.[11]

4. Experimental

4.1. General method and chemicals

Photo irradiation was performed using an Eiko-sha PIH-100 high-pressure mercury lamp (365 nm). Luminescent activity was measured on a lumiphotometer, AB-2200-R (ATTO, Tokyo, Japan).

Coelenterazine was prepared according to Goto's mothod.[12] Apoaequorin solution was prepared from SeaLite Sciences (SeaLite Sciences, Inc., 20 μM, 10 mM Tris–HCl and 10 mM EDTA, 5 mM DTT, pH 7.5). Ethylenediaminetetraacetate dehydrate (EDTA) and dithiothreitol (DTT), trifluoroacetic acid (TFA) were purchased from Nacalai Tesque (Kyoto, Japan). Trypsin (sequencing grade) was purchased from Roche Diagnostic (Mannheim, Germany). 2-Mercaptoethanol (2ME) was purchased from Wako Chemicals (Osaka, Japan). PDB file of aequorin (1EJ3) was downloaded for MacroModel calculation via the Internet at http://www.pdb.org.

4.2. Nano-HPLC-ESI-IT-MS and –MS/MS

Whole LC–MS and –MS/MS experiments were conducted utilizing the house assembled HPLC system (JASCO Co., Ltd. Tokyo, Japan) using Develosil ODS-HG-5 (Nomura Co., Ltd. Aichi, Japan, 150 × 0.3 mm i.d.) columns and measured utilizing an ion trap mass spectrometer Bruker Daltonics HCT Plus (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal ESI source. The columns were equilibrated with 260 μL of water containing 0.025% trifluoro acetic acid at a flow rate of 10 μL/min and then developed using a linear gradient from 0% to 100% of acetonitrile containing 0.025% trifluoro acetic acid for 40 min at a flow rate of 5 μL/min by the PREPACKED-GRADIENT program packed a gradient solvent in a peak tubing (5 m length, 50 μm i.d.), which has a non-split system directly connected to the MS sample inlet, we have performed the whole MS analysis at femto-mol/μl concentration without any loss of samples. The column effluent was monitored by UV at 210 nm and then introduced into the electrospray nebulizer without splitting. MS and MS/MS settings were as follows: the voltage of capillary, 4000 V; drying gas (N_2) flow rate, 5 L/min, dry temperature, 350 °C. An ion trap scanning was performed in the ultra scan mode and the range of mass was *m/z* 50–2000 (target mass was *m/z* 1500). MS/MS scanning was also performed in the ultra scan mode and the range of mass was *m/z* 50–3000. All MS experiments were preformed in positive ion mode. Data were acquired and processed using Compass 1.2 (esquireControlTM and DataAnalysisTM version 3.2) (Bruker Daltonics, Bremen, Germany).

4.3. Incorporation and bioluminescence measurements of coelenterazine into apoaequorin

Incorporation reaction was started by mixing 20 μL of the apoaequorin solution (SeaLite Sciences, Inc., 20 μM, 10 mM Tris–HCl and 10 mM EDTA, 5 mM DTT, pH 7.5), 18 μL of the buffer solution (10 mM Tris–HCl/NaOH buffer, pH 7.5, containing 10 mM EDTA and 10 mM DTT or 20 mM 2ME) and saturated 2 μL of 2 mM coelenterazine (methanolic solution) at 0 °C and then was incubated for 2 h. The light emission of the reconstituted aequorin solution was measured by the addition of 3 mL of 10 mM calcium chloride for 1 min.

4.4. Photo irradiation

Coelenterazine (2 μL of 2 mM) dissolved in methanol was added to the solution of 18 μL of Tris buffer (10 mM Tris–HCl and 10 mM EDTA, 10 mM DTT or 20 mM 2ME, pH 7.5) and 20 μL of the apoaequorin solution (SeaLite Sciences, Inc., 20 μM, 10 mM Tris–HCl and 10 mM EDTA, 5 mM DTT, pH 7.5) in ice bath. The mixture was loaded into any desired position in the glass capillary (ringcaps® 50 μL) and then sealed with gas burner. The sample was incubated for 2 h at 0 °C in the glass capillary and then was irradiated light with a high-pressure mercury lamp (100-W high pressure Hg lamp, 365 nm) for 20 min at room temperature with air. These aequorin solutions were digested by using trypsin dissolved in Tris buffer (20% w/w, 10 mM Tris–HCl and 10 mM EDTA, 5 mM DTT, pH 7.5) and were incubated for 12 h at 37 °C. The resultant solution (10 μL) were injected into nano-HPLC-ESI-IT-MS, -MS/MS.

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