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Oxygen Activation of Apo-obelin–Coelenterazine Complex

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Ca^{2+}-regulated photoproteins use a noncovalently bound 2-hydroperoxycoelenterazine ligand to emit light in response to Ca^{2+} binding. To better understand the mechanism of formation of active photoprotein from apoprotein, coelenterazine and molecular oxygen, we investigated the spectral properties of the anaerobic apo-obelin–coelenterazine complex and the kinetics of its conversion into active photoprotein after exposure to air. Our studies suggest that coelenterazine bound within the anaerobic complex might be a mixture of N7-protonated and C2(−) anionic forms, and that oxygen shifts the equilibrium in favor of the C2(−) anion as a result of peroxy anion formation. Proton removal from N7 and further protonation of peroxy anion and the resulting formation of 2-hydroperoxycoelenterazine in obelin might occur with the assistance of His175. It is proposed that this conserved His residue might play a key role both in formation of active photoprotein and in Ca^{2+}-triggering of the bioluminescence reaction.

Introduction

Ca^{2+}-regulated photoproteins are responsible for light emission in a variety of bioluminescent marine organisms, mostly coelenterates.[1] The best known of these is aequorin isolated from the jellyfish *Aequorea*.[2] All photoproteins known to date consist of a single polypeptide chain (~22 kDa) to which an imidazopyrazinone derivative (2-hydroperoxycoelenterazine) is tightly bound. The light-yielding reaction proceeds at a very slow rate in the absence of Ca^{2+}, but is greatly accelerated upon Ca^{2+} binding.[3] Bioluminescence involves the oxidative decarboxylation of 2-hydroperoxycoelenterazine, thereby generating protein-bound coelenteramide in its excited state.[4] The excited coelenteramide relaxes to its ground state with the production of blue light, with emission maximum around 465–495 nm depending on the source organism.[5]

The main use of Ca^{2+}-regulated photoproteins has been detection of calcium ions in biological systems.[6] Photoproteins have been successfully applied in many different types of living cells, both to estimate intracellular Ca^{2+} concentration under steady-state conditions and to study the role of calcium transients in the regulation of cellular function. Photoproteins were initially injected into cells for these studies or delivered by liposome-mediated transfer, but since the cloning of cDNA genes, expression of recombinant photoproteins within cells is preferred.[6b,c] The success of such photoprotein applications, however, depends on various factors, among which are the rate and efficiency of the generation of active photoprotein from apo-photoprotein, coelenterazine, and oxygen, as well as the influence of the cellular environment on this process.

From determination of different ligand-dependent photoprotein conformational states[7] and the recent studies of photoprotein–GFP interactions,[8] significant insight has been obtained into the in vitro and in vivo bioluminescence mechanism.[5,8d,9] However, much less is known about the mechanism of active photoprotein complex formation from coelenterazine and oxygen. Investigations have dealt with the relative rates of active photoprotein complex formation from wild-type apo-aequorin and coelenterazine (and its analogues), as well as the effect of temperature, pH, incubation time, reducing agent concentrations, and some additives.[10]

Recently, by using intrinsic protein fluorescence quenching, we demonstrated that binding of coelenterazine to the apophotoprotein occurs within milliseconds, in contrast to the formation of active photoprotein complex, which requires minutes to hours.[11] It was shown that the rate-limiting step of active photoprotein formation is the conversion of coelenterazine into its peroxy derivative, which takes place within the substrate-binding cavity. However, what happens to coelenter-

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azine after binding to apo-photoprotein and what coelenterazine intermediates precede 2-hydroperoxycoelenterazine are unknown.

In this study, we explored for the first time the absorption spectral properties of the anaerobic apo-obelin–coelenterazine complex as well as the kinetics of conversion of bound coelenterazine into 2-hydroperoxycoelenterazine upon exposure of the protein–ligand complex to air.

Results and Discussion

Interaction between coelenterazine and apo-obelin

Addition of coelenterazine to apo-obelin resulted in a significant concentration-dependent decrease in intrinsic protein fluorescence.[11] When quenched apo-photoprotein was diluted by a factor of two immediately after coelenterazine addition, an exactly two-fold decrease of protein fluorescence was observed, thus showing that coelenterazine remains tightly associated with apo-photoprotein.[11] The strong association of coelenterazine with apoprotein was attributed to hydrophobic interaction of coelenterazine with nonpolar residues of the coelenterazine-binding cavity. The successful purification of anaerobic apo-obelin–coelenterazine complex by ion-exchange chromatography testifies to the tight complex formation between apo-photoprotein and coelenterazine before coelenterazine modification by oxygen.

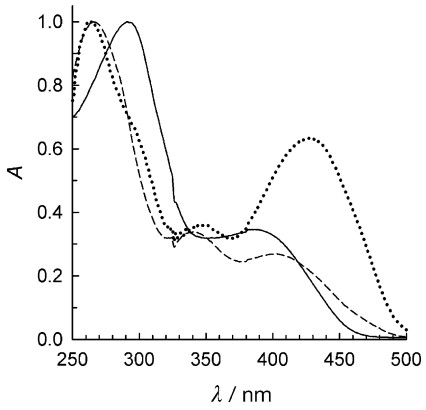
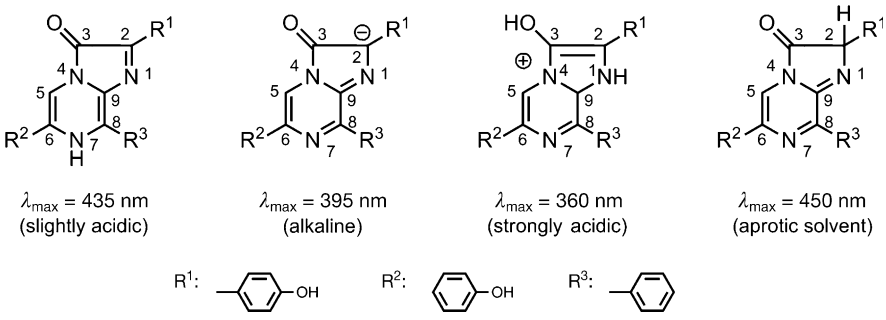


Figure 1. Absorption spectra of coelenterazine at pH 6.5 (····), pH 7.8 (-----), and pH 10.0 (——) under anaerobic conditions. Spectra were measured in Tris·HCl buffer (20 mM, pH 7.8 or 10.0) containing NaCl (0.3 M) and EDTA (5 mM), or in bis-Tris·HCl (50 mM, pH 6.5) containing NaCl (0.3 M), EDTA (5 mM). Coelenterazine concentration was 25 μM.



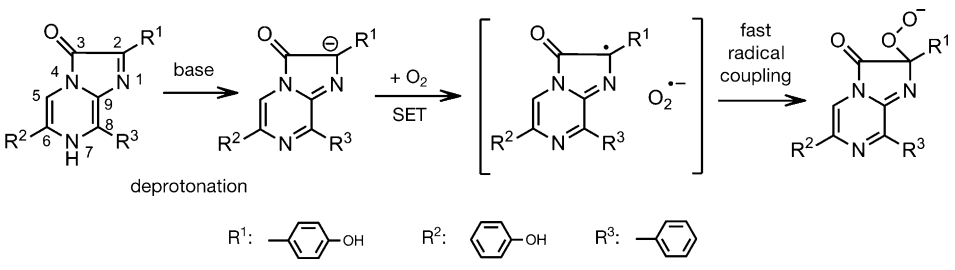
Scheme 1. Tautomeric and ionic forms of coelenterazine in various solvents and at different pH values.[12a]

Absorption spectra of coelenterazine at different pH under anaerobic conditions

In buffers coelenterazine displayed absorption maxima (Figure 1) at ~265, 345, and 430 nm (pH 6.5), at ~268, 340, and 410 nm (pH 7.8), and at 290 and ~390 nm (pH 10.0). The spectral variation shows that the ionization state of coelenterazine alters in response to pH changes. Based on earlier studies of solvent- and pH-dependent spectral properties of coelenterazine derivatives (Scheme 1), the absorption spectra at pH 6.5 and 10.0 can be attributed to protonated N7 and C2(−) anionic forms of coelenterazine.[12] As the absorption spectrum of coelenterazine at pH 7.8 resembles a superposition of the spectra at pH 6.5 and 10.0, it is reasonable to assume that under anaerobic conditions at pH 7.8 coelenterazine is present as a mixture of protonated N7 and C2(−) anionic forms (Figure 1).

Reaction of free coelenterazine with oxygen

Coelenterazine emits light in the presence of oxygen when dissolved in aprotic solvents containing a trace amount of a base. It was suggested that the first step in this process involves deprotonation of N7 of coelenterazine with a base to yield its C2(−) anion.[13] Then, oxygen becomes bound at the C2-position of coelenterazine, thus yielding the peroxide anion (Scheme 2), which, following cyclization, leads to formation of dioxetanone. The latter species then promptly decomposes with release of CO_2, thereby generating the singlet excited state of the amide product.[13c]



Scheme 2. Proposed mechanism of formation of coelenterazine peroxide anion.[13c]

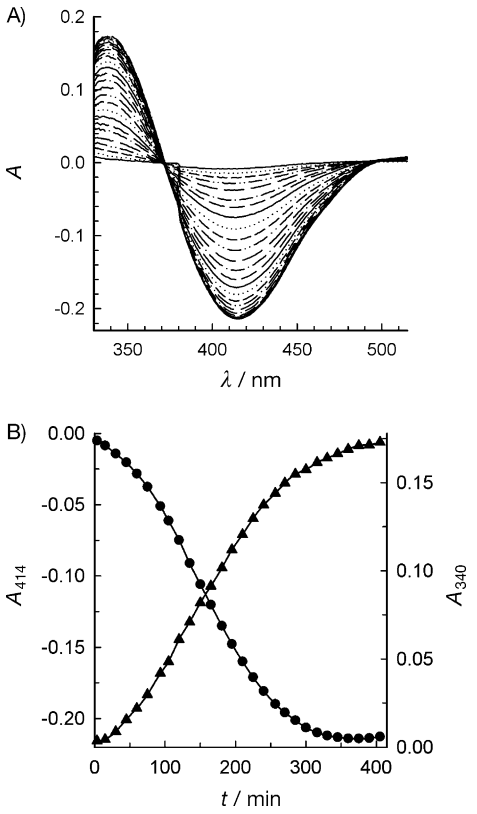


Figure 2. Change in coelenterazine tautomeric form in the presence of oxygen at pH 7.8. A) Difference absorption spectra, and B) kinetics of coelenterazine conversion into product monitored at 340 nm (▲) and 414 nm (●). Spectra were measured over 435 min from sample (open cuvette) against anaerobic sample (sealed cuvette) every 5 min for the first 30 min, and then every 15 min. Before taking the first spectrum, the sample was vigorously mixed with air by pipetting in the cuvette for several minutes to avoid oxygen-diffusion limiting conditions. Coelenterazine concentration was 25 μM.

Figure 2 shows the changes in the absorption spectrum of coelenterazine at pH 7.8 in response to oxygen. After exposure to air, the coelenterazine solution displayed a gain in absorption at ~340 nm which, in fact, corresponded to appearance of the chemiluminescence reaction product, coelenteramide, and to disappearance of the absorption maximum at 414 nm, which corresponds to a decrease in some tautomeric form of coelenterazine at pH 7.8 (Scheme 1). It should be noted that the kinetics of the absorption changes in Figure 2 are sigmoidal.

The protonated tautomeric form of coelenterazine, which is present in solution at pH 6.5 (according to spectral data, Figure 1), also reacts with oxygen. When anaerobic coelenterazine was exposed to air at pH 6.5, absorption again increased at ~340 nm but decreased at 430 nm (data not shown).

Spectral characterization of anaerobic apo-obelin–coelenterazine complex

Apo-obelin has a UV/Vis absorption spectrum that is typical of proteins without an organic ligand, with a maximum at 280 nm and a shoulder at 295 nm.[11] Active obelin (that is, with bound 2-hydroperoxycoelenterazine) displays in addition an absorption maximum at ~470 nm and a shoulder at 310 nm.[11]

The absorption spectrum of the anaerobic apo-obelin–coelenterazine complex displayed an extra absorption maximum at 355 nm and a shoulder at ~400 nm; this can be attributed to bound coelenterazine (Figure 3 A). Under anaerobic conditions in a sealed cuvette the spectrum did not significantly change over several hours (Figure 3 A).

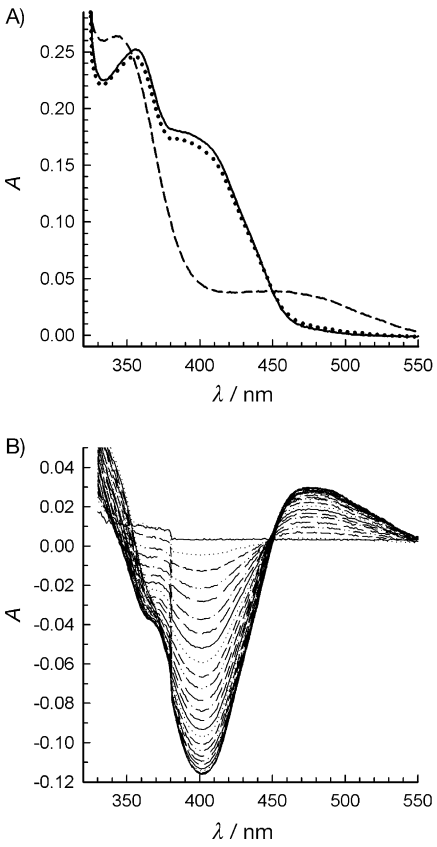


Figure 3. Absorption spectra of apo-obelin–coelenterazine complex. A) Absorption spectra of anaerobic apo-obelin–coelenterazine complex in sealed cuvette (——), anaerobic apo-obelin–coelenterazine complex in sealed cuvette after 435 min (····), and active obelin after 435 min (-----). B) Difference spectra showing the kinetics of conversion of apo-obelin–coelenterazine complex into active obelin after exposure to air. Sample preparation and recording as described in Figure 2. Anaerobic complex concentration was 45 μM.

Kinetics of conversion of apo-obelin–coelenterazine complex into active photoprotein

Aerated apo-obelin–coelenterazine complex has an absorption spectrum with a maximum at ~470 nm, thus indicating the presence of bound 2-hydroperoxycoelenterazine (Figure 3 A). Difference spectra were recorded to compare absorption of the apo-obelin–coelenterazine complex upon activation by oxygen (open cuvette) with that of an anaerobic sample (sealed cuvette). In the course of conversion, the shoulder at 400 nm disappeared, the maximum at 355 nm shifted to 345 nm, and a new absorption band appeared at ~470 nm,

characteristic of 2-hydroperoxycoelenterazine (Figure 3B). The apparent rate constants calculated from the absorption changes at 400 and 470 nm were $k_{400\,nm} = (5.3 \pm 0.3) \times 10^{-3}$ min^{-1} and $k_{470\,nm} = (8.6 \pm 0.6) \times 10^{-3}$ min^{-1}.

To exclude the potential limiting effect of oxygen diffusion on the rate of active photoprotein formation, we also performed an alternative oxygen-activation experiment. For this, a concentrated anaerobic sample of apo-obelin–coelenterazine complex was diluted ten times in air-saturated buffer and extensively mixed. After that, absorption spectra were recorded against buffer (Figure 4A).

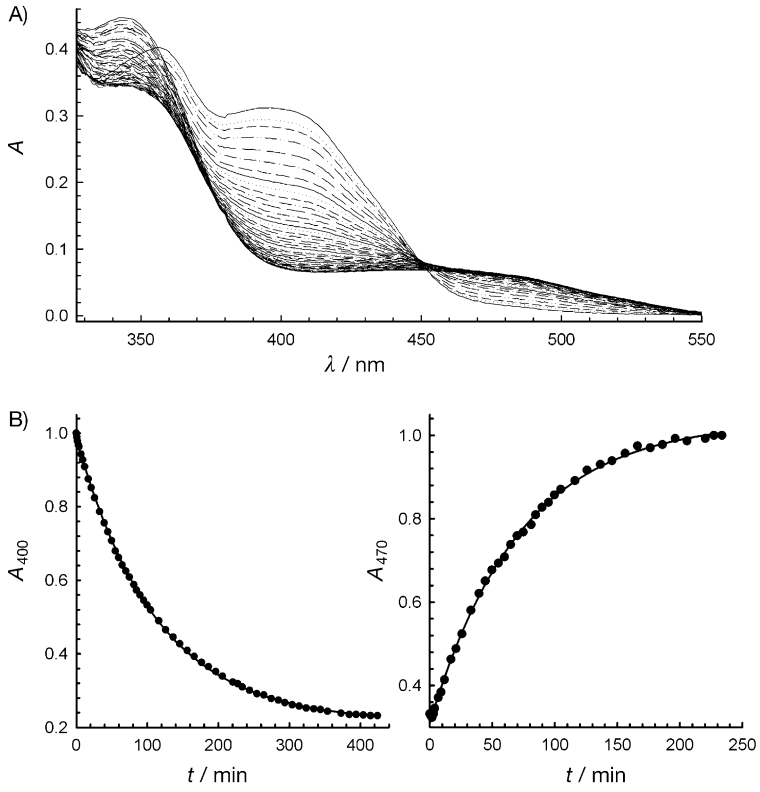


Figure 4. Reaction of anaerobic apo-obelin–coelenterazine complex with oxygen. A) Change in absorption spectrum of air saturated sample every minute for 10 min then every 10 min until 430 min; B) Kinetics of the complex conversion into active photoprotein monitored at 400 nm (left) and at 470 nm (right). Anaerobic complex concentration was 68 μM.

The spectra clearly demonstrate that there was no significant difference between the two oxygen-activation experiments: in both cases (Figures 3 and 4A) the shoulder at 400 nm disappears over time, the maximum at 355 nm shifts to 345 nm, and a new absorption band appears at ~470 nm. However, the $k_{400\,nm}$ and $k_{470\,nm}$ apparent rate constants estimated from the second experiment (9.0 ± 0.05 and $14.0 \pm 0.3 \times 10^{-3}$ min^{-1} respectively, Figure 4B) exceeded those calculated from data shown in Figure 3B. This indicates the dependence of apparent rate on oxygen concentration. Note that the $k_{470\,nm}/k_{400\,nm}$ ratio is approximately the same in both cases, that is, the apparent rate constants calculated from the absorption changes at 470 nm are ~1.5–1.6 times higher than those determined from the absorption changes at 400 nm.

As the increase in the absorption at 470 nm corresponds to accumulation of 2-hydroperoxycoelenterazine in the substrate-binding cavity of obelin, we compared the kinetics of active obelin formation monitored by absorbance at 470 nm with the parameters determined by bioluminescence (Figure 5). A concentrated anaerobic sample of apo-obelin–coelenterazine complex was diluted ten times in air-saturated buffer, extensively mixed, and then bioluminescence kinetics were measured upon injection of calcium solution into sample aliquots at different incubation times. The apparent rate constant of bioluminescence formation (k_{BL}) was evaluated as $24.7 \pm 1.4 \times 10^{-3}$ min^{-1}, which is somewhat higher than that calculated from the absorption changes at 470 nm ($14.0 \pm 0.3 \times 10^{-3}$ min^{-1}).

Assignment of coelenterazine derivative bound within anaerobic apo-obelin–coelenterazine complex

Comparison of the spectral properties of free coelenterazine and the apo-obelin–coelenterazine complex at pH 7.8 allowed us to speculate on the molecular structure of the coelenterazine derivative bound within the anaerobic complex. Although not identical, the absorption spectrum of free coelenterazine at pH 7.8 (Figure 1) is very similar to that of the anaerobic apo-obelin–coelenterazine complex (Figure 3A). The maximum (at ~410 nm) observed in the absorption spectrum of free anaerobic coelenterazine is shifted (400 nm) for the apo-obelin–coelenterazine complex. As the absorption spectrum of coelenterazine at pH 7.8 (as had been assumed) represents a mixture of protonated and ionic forms, we propose that coelenterazine bound within the anaerobic complex might be in equilibrium between N7(H) and C2(−) forms.

During the conversion of apo-obelin–coelenterazine complex into active obelin, two main absorption changes were observed: the shoulder at 400 nm dis-

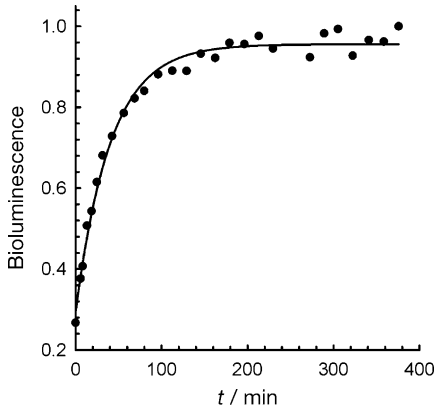


Figure 5. Kinetics of apo-obelin–coelenterazine complex activation by oxygen monitored by bioluminescence. Anaerobic complex concentration was 74 μM.

appeared (Figure 3B), which can be attributed to diminution of coelenterazine derivative bound within the anaerobic apo-obelin–coelenterazine complex; and a new absorption band characteristic of 2-hydroperoxycoelenterazine appeared (~470 nm). The $k_{400\,nm}$ and $k_{470\,nm}$ apparent rate constants differed the two cases (Figure 3B and 4B). However, the $k_{470\,nm}/k_{400\,nm}$ ratio was independent of oxygen concentration, that is, the apparent rate constants calculated from the absorption changes at 470 nm were ~1.5–1.6 times higher than those for the absorption changes at 400 nm. As coelenterazine bound within the anaerobic complex might be a mixture of C2(−) anionic and N7-protonated forms and $k_{400\,nm}$ is slower than $k_{470\,nm}$, we can assume that both steps are oxygen dependent and that formation of 2-hydroperoxycoelenterazine might shift the equilibrium between initial species.

Photoprotein reaction with oxygen is rather slow,[10a] and evidently depends on the protein preparation procedure and reaction conditions. In contrast, many other monooxygenases and oxidases typically react very rapidly with oxygen.[14] The conversion of apo-obelin–coelenterazine complex into photoprotein depends on oxygen concentration, so diffusion of O_2 to its target binding site might be the rate-determining factor. Although oxygen diffusion in water is sufficiently fast,[15] at the enzymes oxygen diffusion through protein channels can be significantly hindered by steric constraints.[16] In the case of the apo-obelin–coelenterazine complex oxygen diffusion might be further restricted by the absence of discrete oxygen channels to the internal coelenterazine binding site.[7a–c] Thus, generation of active obelin involves rapid binding and re-equilibration of coelenterazine tautomeric forms, followed by slow insertion of molecular oxygen to yield 2-hydroperoxycoelenterazine.

His 175 as a possible proton shuttle

As active photoprotein is formed at neutral pH from apoprotein, coelenterazine, and oxygen, it is likely that an active-site residue might act as an acid or base in 2-hydroperoxycoelenterazine formation. According to the crystal structures of obelin and aequorin,[7a–c] His175 in obelin (His169 in aequorin) is situated in the C-terminal helix, which interacts with the N-terminal helix closing the substrate-binding cavity, not far from the C2-atom of coelenterazine (Figure 6). This His residue is crucial for photoprotein bioluminescence because its substitution to Ala, Phe, or Trp led to complete loss of bioluminescence activity, whereas modification of other histidines yielded mutant photoproteins with varying bioluminescence activity.[17]

For several cofactor-independent oxidases and oxygenases, including *Renilla* luciferase, substrate activation for subsequent insertion of oxygen was also proposed to involve a histidine.[18] In these enzymes, the histidine plays a key role in the proton relay system by acting as a general base and proton donor/acceptor. In Ca^{2+}-regulated photoproteins His might carry out a similar function, by subtracting a proton from N7 of coelenterazine upon its fast binding to apo-photoprotein, yielding the coelenterazine C2(−) anion, which further reacts with oxygen yielding the peroxide anion (Scheme 2). This would be followed by conformation transition in the protein molecule giving this proton back, yielding 2-hydroperoxycoelenterazine and consequently active photoprotein in its final conformation (Figure 6).

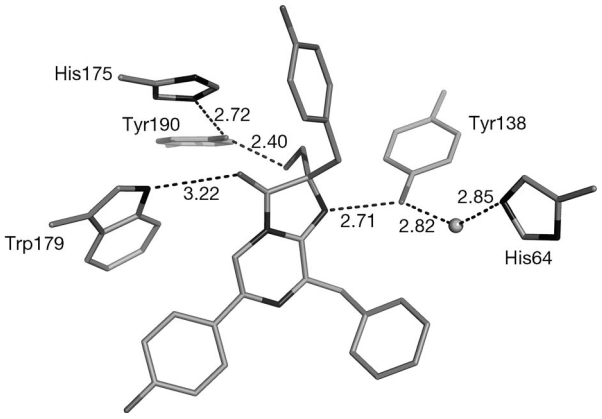


Figure 6. Residues surrounding 2-hydroperoxycoelenterazine in obelin (PDB ID: 1QV0). The gray ball represents a water molecule. Hydrogen bonds (-----) were determined with the PyMOL program. Distances are in Å.

Conclusions

In summary, this study brings further insight into the mechanism of active photoprotein-complex formation from apoprotein, coelenterazine, and oxygen. We show for the first time the formation of a tight complex between apo-photoprotein and coelenterazine in the absence of oxygen. Our spectroscopic studies suggest that coelenterazine bound within the anaerobic complex might be a mixture of N7-protonated and C2(−) anionic forms. Proton removal from N7 and further reprotonation of C2 peroxide anion might occur with the assistance of histidine, thus implying that this residue plays a key role both in active photoprotein formation and in Ca^{2+} triggering of photoprotein bioluminescence.

Experimental Section

Chemicals: Unless stated otherwise, chemicals were from Sigma–Aldrich at the purest grade available. High purity coelenterazine was purchased from JNC Corporation (Yokohama, Japan).

Protein expression and purification: Apo-obelin was expressed and purified as previously reported.[19] For protein production, transformed *Escherichia coli* BL21 Gold was cultivated with vigorous shaking at 37 °C in lysogeny broth (LB) medium containing ampicillin (200 $\mu g\,mL^{-1}$), and was induced with IPTG (0.5 mM) when the culture reached $OD_{600} = 0.5–0.6$. After addition of IPTG, the cultivation was continued for 3 h. Most of the produced apo-obelin accumulated as inclusion bodies. The apo-photoprotein was dissolved in urea (6 M), purified on a DEAE Sepharose Fast Flow column (GE Healthcare) with urea (6 M), and concentrated by ultracentrifugal filtration using Amicon Ultra Centrifugal Filters (Millipore).

Apo-obelin concentration was determined spectrophotometrically using $\varepsilon_{280} = 40\,450\,M^{-1}\,cm^{-1}$ calculated with the ProtParam tool (http://us.expasy.org/tools/protparam-doc.html), which uses Edelhoch's method.[20] The concentration of coelenterazine in the etha-

nol stock solution was determined from its molar absorption coefficient ($\varepsilon_{435} = 9800\ M^{-1}\,cm^{-1}$).[21]

Preparation of the anaerobic apo-obelin–coelenterazine complex: To remove oxygen, solutions were degassed by subjecting them to cycles of vacuum treatment and nitrogen flushing (×10–15). All steps in the preparation of the apo-obelin–coelenterazine complex were carried out within a Forma Anaerobic Station (Thermo Fisher). First, solutions were placed into the anaerobic chamber and tested for the presence of oxygen. The conversion of apo-obelin into active photoprotein serves as an indicator of oxygen, as oxygen is required for bioluminescence of obelin. Aliquots (15 μL) of degassed solution within the chamber (i.e., chromatography buffers, calcium solution, activation buffer) were mixed with concentrated apo-obelin (5 μL) and charging buffer (5 μL, EDTA (5 mM), DTT (10 mM), Tris·HCl (20 mM, pH 7.0)) containing coelenterazine (50 μM). After 15 min of incubation, Ca^{2+} solution (2 μL, $CaCl_2$ (100 mM), Tris·HCl (100 mM, pH 7.0)) was injected. Lack of visible light indicated that the degassing procedure had been successful.

To produce the anaerobic apo-obelin–coelenterazine complex, degassed apo-obelin (~2 mL, 2 mg mL^{-1}) in urea (6 M) was diluted (÷10) in buffer (EDTA (5 mM), DTT (10 mM), Tris·HCl (20 mM, pH 7.0)) containing coelenterazine (1.1 molar excess to protein) and kept in an anaerobic chamber overnight. The following steps were also performed in the anaerobic station. To remove free apo-obelin and unbound coelenterazine,[18b] the sample was loaded on a degassed Q Sepharose Fast Flow column (160×10 mm) equilibrated with EDTA (5 mM) and Tris·HCl (20 mM, pH 7.0). After washing with 2 column volumes of starting buffer, the apo-obelin–coelenterazine complex was eluted with NaCl (300 mM) in the same buffer. The fraction with a yellowish color was collected and concentrated by ultracentrifugal filtration. For absorption measurements, a purified protein sample was placed into special sealable quartz cuvette (#117.104-QS; Hellma Analytics, Müllheim, Germany) and tightly sealed to prevent oxygen contamination. Finally, samples were removed from the anaerobic station for further measurements.

Anaerobic apo-obelin–coelenterazine complex was produced only from apo-obelin in urea (6 M). Attempts to obtain oxygen-free complex from refolded apo-obelin failed.

Spectral measurements: Absorption spectra of the apo-obelin–coelenterazine complex in the absence and presence of oxygen were obtained with a U-2010 double-beam spectrophotometer (Hitachi). Absorption spectra of the apo-obelin–coelenterazine complex were measured relative to NaCl (0.3 M), EDTA (2 mM), and Tris·HCl (20 mM, pH 7.8). Coelenterazine was dissolved in NaCl (0.3 M), EDTA (5 mM), and Tris·HCl (20 mM, pH 7.8 or 10.0), and in NaCl (0.3 M), EDTA (5 mM), bis-Tris·HCl (50 mM, pH 6.5). The pH of the solution was always checked before coelenterazine addition and adjusted if necessary. The ethanol concentration in the final solution never exceeded 1% (*v*/*v*). Difference absorption spectra of the apo-obelin–coelenterazine complex and coelenterazine were obtained by measuring absorption of the sample in the open cuvette with an excess of air against a sealed sample (containing complex or coelenterazine) every 5 min over 30 min, and then every 15 min over 400 min, at 25 °C.

Bioluminescence measurements: Bioluminescence of obelin during its activation was measured with a Varioskan Flash luminometer (Thermo Scientific). Bioluminescence was triggered by rapid injection of 190 μL of $CaCl_2$ (100 mM) and Tris·HCl (20 mM, pH 7.0) into photoprotein solution (10 μL) of the anaerobic apo-obelin–coelenterazine complex, rapidly premixed with NaCl (0.3 M), EDTA (2 mM), Tris·HCl (20 mM, pH 7.8) and excess air. The bioluminescence signal was integrated over 4 s at 25 °C.

Acknowledgements

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Keywords: aequorin · coelenterazine · luminescence · photoprotein · protein folding

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