Journal of Photochemistry and Photobiology B: Biology 127 (2013) 133–139

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Role of key residues of obelin in coelenterazine binding and conversion into 2-hydroperoxy adduct

Elena V. Eremeeva [a,b,c], Svetlana V. Markova [a,c], Willem J.H. van Berkel [b], Eugene S. Vysotski [a,c,*]

[a] Photobiology Laboratory, Institute of Biophysics, Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia
[b] Laboratory of Biochemistry, Wageningen University, 6703 HA Wageningen, The Netherlands
[c] Laboratory of Bioluminescence Biotechnology, Institute of Fundamental Biology and Biotechnology, Siberian Federal University, Krasnoyarsk 660041, Russia

ARTICLE INFO

Article history:
Received 2 July 2013
Received in revised form 10 August 2013
Accepted 20 August 2013
Available online 28 August 2013

Keywords:
Bioluminescence
Coelenterazine
Obelin
Aequorin
Photoprotein

ABSTRACT

Bioluminescence of a variety of marine organisms is caused by monomeric Ca^{2+}-regulated photoproteins, to which a peroxy-substituted coelenterazine, 2-hydroperoxycoelenterazine, is firmly bound. From the spatial structure the side chains of Tyr138, His175, Trp179, and Tyr190 of obelin are situated within the substrate-binding pocket at hydrogen bond distances with different atoms of the 2-hydroperoxycoelenterazine. Here we characterized several obelin mutants with substitutions of these residues regarding their bioluminescence, coelenterazine binding, and kinetics of active obelin formation. We demonstrate that Tyr138, His175, Trp179, and Tyr190 are all important for coelenterazine activation; substitution of any of these residues leads to significant decrease of the apparent reaction rate. The hydrogen bond network formed by Tyr138, Trp179 and Tyr190 participates in the proper positioning of coelenterazine in the active site and subsequent stabilization of the 2-hydroperoxy adduct of coelenterazine. His175 might serve as a proton shuttle during 2-hydroperoxycoelenterazine formation.

1. Introduction

Bioluminescence is a widely distributed phenomenon among marine dwellers [1,2]. Many of these luminous organisms generate light by oxidation of coelenterazine, an imidazopyrazinone derivative [3,4]. The chemical mechanism of light emission appears to be common among coelenterazine utilizing organisms but often differs in the detailed biochemical process, probably as necessitated by the behavioral function of bioluminescence [5].

The Ca^{2+}-regulated photoproteins constitute one specific class of coelenterazine utilizing molecules. They comprise monomeric polypeptides with a molecular mass of ~22 kDa to which a peroxy-substituted coelenterazine, 2-hydroperoxycoelenterazine, is tightly but non-covalently bound. Since Ca^{2+}-regulated photoproteins contain a stable oxygenated reaction intermediate, they are capable of emitting light in proportion to the concentration of protein unlike the luciferases where the amount of light emitted is proportional to the concentration of the substrate luciferin [6]. Bioluminescence is triggered upon binding of Ca^{2+}, which induces the oxidative decarboxylation of 2-hydroperoxycoelenterazine with generation of protein-bound reaction product, coelenteramide, in its excited state [7,8]. The excited coelenteramide relaxes to its ground state with the production of blue light with maxima around 465–495 nm depending on the type of photoprotein [9].

Although Ca^{2+}-regulated photoproteins have been detected in many different marine organisms [3,10], cDNA sequence information is only available for five hydromedusan photoproteins, namely aequorin [11–13], mitrocomin (halistaurin) [14], and clytin (phialidin) [15–17] from the jellyfishes *Aequorea, Mitrocoma* (*Halistaura*), and *Clytia* (*Phialidium*), and obelins from the hydroids *Obelia longissima* [18,19] and *Obelia geniculata* [20], and for the light-sensitive photoproteins from ctenophore *Beroe abyssicola* [21], *Mnemiopsis leidyi* [22,23], and *Bathocyroe fosteri* [24]. All Ca^{2+}-regulated photoproteins contain three calcium-binding consensus sequences characteristic of EF-hand Ca^{2+}-binding proteins [25,26]. Apophotoproteins expressed in *Escherichia coli* can be converted to active photoproteins by incubating them with coelenterazine under Ca^{2+}-free conditions in the presence of O_2 and reducing agents [27].

The main application of Ca^{2+}-regulated photoproteins originates from their ability to emit light upon Ca^{2+} binding. Photoproteins have been successfully used to estimate the intracellular Ca^{2+} concentration under steady-state conditions and to study the role of calcium transients in the regulation of cellular function in different types of cells [28]. The cloning of cDNAs encoding apophotoproteins has granted a new approach in photoprotein applications. The recombinant apophotoprotein expressed intracellularly forms

* Corresponding author at: Photobiology Laboratory, Institute of Biophysics, Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia. Tel.: +7 3912494430; fax: +7 391433400.
E-mail address: eugene.vysotski@gmail.com (E.S. Vysotski).

1011-1344/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jphotobiol.2013.08.012

the active photoprotein upon binding with coelenterazine, which is added externally and diffuses into the cell. Thus, such cells have, in effect, a "built-in" calcium indicator. This approach is highly valuable because it does not require laborious procedures like microinjection or liposome-mediated transfer and now is widely used [29–31]. The success of such photoprotein applications, however, depends on various factors among which are the rate and efficacy of the generation of active photoprotein from apophotoprotein, coelenterazine, and oxygen, as well as any influence of the cellular environment on these processes.

In the past decade the crystal structures of photoproteins aequorin [32], obelin [33,34], and clytin [35] as well as obelin ligand-dependent conformation states [36–38] have been determined. Based on these findings and mutagenesis studies of some substrate-binding cavity residues [39–42] significant insight has been obtained into the bioluminescence mechanism [9,38,42,43]. At the same time much less is known about the molecular mechanism of active photoprotein complex formation from apophotoprotein, coelenterazine and oxygen (Scheme 1). Previous studies focused primarily on the efficiency of regeneration of wild type apo-aequorin with coelenterazine derivatives as well as the effects of temperature, pH, incubation time and some additives on this process [27,44–46]. It was shown, for example, that dithiothreitol (DTT) or β-mercaptoethanol are required to reduce disulfide bonds in the recombinant apo-aequorin [47]. Furthermore, it was determined that the binding of coelenterazine to apophotoprotein occurs within milliseconds [48], in contrast to the formation of active photoprotein complex [27]. This finding evidently showed that the rate-limiting step of active photoprotein formation is the conversion of coelenterazine into its peroxy derivative.

The substrate-binding cavity of obelin is highly hydrophobic with addition of several hydrophilic side chains (His22, Tyr138, His175, and Tyr190) directed internally. The side chains of Tyr138, His175, Trp179, and Tyr190 are situated within the coelenterazine-binding pocket at hydrogen bond distances with certain atoms of the 2-hydroperoxycoelenterazine (Fig. 1). Recently, it was shown that the hydrogen bond network formed by His175–Trp179–Tyr190 triad participates in positioning and stabilizing the 2-hydroperoxy adduct of coelenterazine and that His175 is critical for the bioluminescence function [49]. In active obelin His175, Trp179, and Tyr190 are located near the C2 atom of coelenterazine, which is the exact position for oxygen to react with the substrate. Thus, it looks reasonable to assume that these residues could be involved in the process of active photoprotein complex formation. Tyr138 is hydrogen bonded to the N1 atom of 2-hydroperoxycoelenterazine (Fig. 1) and potentially might effect the charge distribution in the imidazopyrazinone ring of the coelenterazine molecule via polarizing the N1-nitrogen and thus the efficiency of the coelenterazine reaction with oxygen.

In this paper we report for the first time the study of the function of amino acid residues located in the substrate-binding cavity of Ca^{2+}-regulated photoprotein obelin in the formation of 2-hydroperoxycoelenterazine and consequently active obelin. Several obelin mutants with substitutions of Tyr138, His175, Trp179 and Tyr190 were characterized regarding their bioluminescence, coelenterazine binding and kinetics of active obelin formation.

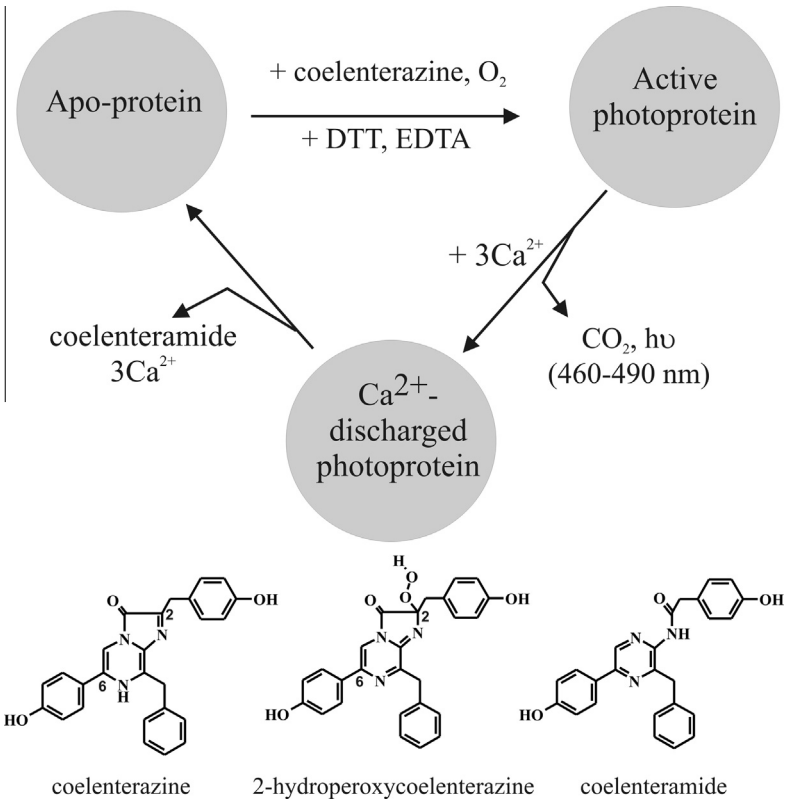
2. Experimental methods

2.1. Materials

Coelenterazine was obtained from Prolume Ltd. (Pinetop, AZ, USA). Other chemicals, unless otherwise stated, were from Sigma–Aldrich and the purest grade available.

2.2. Molecular biology

Site-directed mutagenesis was done on the template pET19-OL8 *E. coli* expression plasmid carrying the *O. longissima* wild type apo-obelin gene [51]. Mutations resulting in the desired amino



Scheme 1.

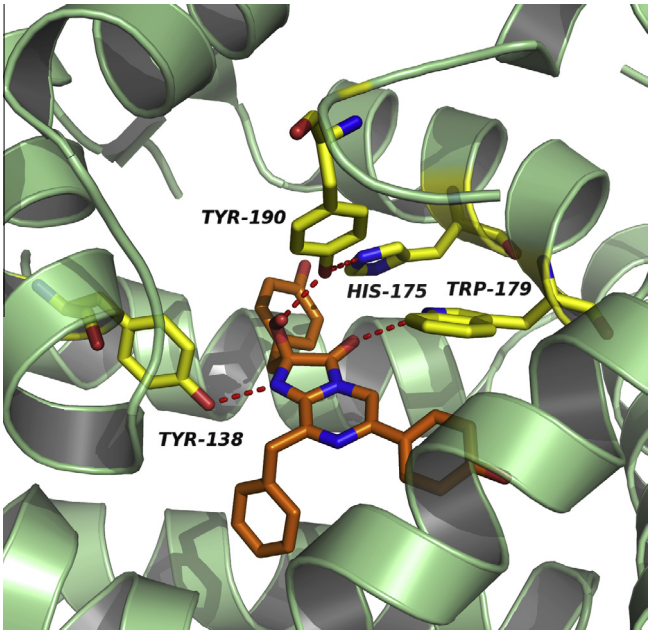


Fig. 1. Hydrogen binding mode of 2-hydroperoxycoelenterazine with surrounding residues in obelin (PDB code 1QV0). Hydrogen bonds (dashed lines) are determined with the PyMOL program [50].

acid change were carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the protocol supplied with the kit. The plasmids harboring the mutations were verified by DNA sequencing.

2.3. Apophotoprotein preparation

Apo-obelin and its mutants were expressed and purified as previously reported [49,52]. The apophotoproteins obtained after extraction with 6 M urea and purification on a DEAE-Sepharose Fast Flow column were concentrated by Amicon Ultra Centrifugal Filters (Millipore). To fold apophotoproteins, the concentrated samples containing 6 M urea were diluted approximately 20-fold with a solution containing 1 mM EDTA, 20 mM Tris–HCl pH 7.0, again concentrated, and then washed several times with the same buffer to remove any impurities of urea and salts. The apophotoproteins were centrifuged (20,000$g \times 10$ min) at 4 °C, incubated overnight with 10 mM DTT, again centrifuged, and then passed through a Superdex 200 column (Amersham Bioscience) equilibrated with freshly prepared 10 mM DTT, 5 mM EDTA, 20 mM Tris–HCl pH 7.0 to produce monomeric apophotoprotein containing no disulfide bonds and aggregates [49]. The final preparations of apophotoproteins were homogeneous according to SDS–PAGE and gel filtration. The active wild type obelin and its mutants were produced as described elsewhere [52,53]. The concentrations of apo-obelin and its mutant were determined using the corresponding molar extinction coefficients at 280 nm calculated with the ProtParam tool (http://us.expasy.org/tools/protparam-doc.html) that uses Edelhoch's method [54].

2.4. Fluorescence measurements and determination of apparent dissociation constant of the apophotoprotein-coelenterazine complex

Fluorescence measurements were carried out with a Varian Cary Eclipse spectrofluorimeter (Agilent Technologies, USA) in 5 mM EDTA, 10 mM DTT, 20 mM Tris–HCl pH 7.0 at 20 °C. Excitation was at 295 nm (slit 5 nm). The apo-obelin fluorescence emission spectra were corrected with the computer program supplied with the instrument. All spectra were taken using a standard quartz cuvette (1×1 cm) in a 1-mL initial volume with varied coelenterazine additions in 5- to 10-μL portions up to saturation. To assess fluorescence quenching only the changes of fluorescence intensity at 336 nm were used. The fluorescence intensities were corrected for dilution due to the addition of coelenterazine, for the methanol influence on Trp fluorescence, scattered light, and for inner filter effects of protein and added coelenterazine. To evaluate the inner filter effects, absorbance measurements were performed at excitation and emission wavelengths and fluorescence (F) was corrected using the equation:

$$F = F_{unc} e^{\frac{A_{295}+A_{336}}{2}} \quad (1)$$

where A_{295} and A_{336} are absorbance of protein and ligand at excitation and emission wavelengths, respectively, and F_{unc} is the uncorrected fluorescence.

The apparent dissociation constant of the apophotoprotein-coelenterazine complex was determined using the quenching of apophotoprotein Trp fluorescence upon binding to coelenterazine. Analysis assumes that the fraction of bound ligand is equal to the ratio of the fluorescence quenching ($Q = F_o - F_q$) to maximum quenching ($Q_{max} = F_o - F_{qmax}$), where F_o, F_q, and F_{qmax} are fluorescence intensity at 336 nm measured in the absence of added ligand, the quenched fluorescence intensity in the presence of ligand, and the maximum fluorescence quenching at a saturating level of ligand, respectively. The apparent dissociation constants were calculated by fitting the relative fluorescence emission to Eq. (2), a modified equation compared with the one described elsewhere [55]:

$$\frac{Q}{Q_{max}} = \frac{(C + L + K_D) - \sqrt{(C + L + K_D)^2 - 4CL}}{2} \quad (2)$$

where C, L, and K_D are apophotoprotein and coelenterazine concentrations, and apparent dissociation constant, respectively.

2.5. Measurements of bioluminescence and kinetics of apophotoprotein activation with coelenterazine

The total bioluminescence of obelin and its mutants was measured using a plate luminometer Mithras LB 940 (Berthold, Germany) by injection of 50 μL of 100 mM $CaCl_2$, 100 mM Tris–HCl, pH 8.8 into the well containing 100 μL of 5 mM EDTA, 100 mM Tris–HCl pH 8.8 and the photoprotein aliquot. The bioluminescence signal was integrated during 10 s.

At studies of apophotoprotein activation kinetics the light emission was measured with a BLM 8801 photometer (SCTB "Nauka", Krasnoyarsk, Russia) equipped with the temperature-stabilized cuvette block and neutral-density filters with different transmission coefficients to extend the dynamic range. The kinetics was studied at concentrations of apophotoprotein and coelenterazine equal to 50 nM and 750 nM respectively in air-saturated buffer 5 mM EDTA, 10 mM DTT, 20 mM Tris–HCl pH 6.5. Coelenterazine was added from a concentrated stock solution in methanol. To eliminate any effect of methanol on apophotoprotein activation, the methanol concentration in activation buffer never exceeded 1% (v/v). The coelenterazine concentration in the methanol stock solution was determined spectrophotometrically using the molar absorption coefficient $\varepsilon_{435\ nm} = 9800\ M^{-1}\ cm^{-1}$ in methanol [10]. The bioluminescence was triggered by forceful injection of 10 μL aliquot of the mixture containing apophotoprotein and coelenterazine taken from the activation solution into the cuvette of the luminometer containing 490 μL of 10 mM $CaCl_2$, 100 mM Tris–HCl pH 8.8. The measurements were carried out at 20 °C.

3. Results

3.1. Coelenterazine binding with mutant apo-obelins

Most obelin mutants with replacement of either His175, Trp179, or Tyr190 display very low or no bioluminescent activity [49]. To test the function of these residues in formation of active photoprotein complex the most active mutant from each group (H175 N, Y190F, and W179F [49]) was selected. To reveal the function of Tyr138, mutant Y138F [38] was taken because this mutant retains decent bioluminescent activity and evidently does not form a hydrogen bond with the N1 atom of 2-hydroperoxycoelenterazine. Additionally, we constructed the Y138 W mutant because, according to the crystal structure of wild type obelin, there is enough space at position 138 for a Trp side chain and because its amide nitrogen might form a hydrogen bond with the N1 atom of 2-hydroperoxycoelenterazine.

Addition of coelenterazine to apo-obelin induces a strong concentration-dependent decrease of tryptophan fluorescence [48]. This property was used to determine the apparent dissociation constants of the mutant obelin-coelenterazine complexes. The tryptophan fluorescence quenching data were fitted according to Eq. 2 (Fig. 2).

The apparent dissociation constants for H175N and Y190F mutants turned out to be almost equal to that of wild type apo-obelin (~0.2 μM). A lowered affinity for coelenterazine was observed for Y138F, W179F and especially Y138W (Table 1). For the latter mutant, the apparent dissociation constant amounts to 1.81 ± 0.2 μM which is almost one order of magnitude higher than for wild type obelin.

3.2. Kinetics of active obelin complex formation

Recombinant apophotoproteins can be converted to the active photoproteins by incubating with coelenterazine under calcium-free conditions in the presence of oxygen and reducing agents like DTT or β-mercaptoethanol [27]. It was proposed that the reducing agent is needed to prevent the formation of disulfide bonds in recombinant apophotoproteins which might lead to conformational constraints hindering coelenterazine binding [47]. The thiol-reducing agents are not strictly required for the formation of active photoprotein because an aequorin mutant with all three cysteines replaced for serine is converted into active photoprotein in the absence of these agents [56,57].

We found that at 20 °C and pH 6.5 the concentration of active obelin reaches a maximum after 15 min of incubation with coelenterazine (Fig. 3), instead of several hours as reported before [27]. This essential shortening of activation time is conditioned by the method of apophotoprotein preparation. In our studies, the apo-obelin was pre-incubated with DTT overnight and then its monomeric form was isolated by gel filtration on a Superdex 200 column. This procedure allows the removal of apophotoprotein aggregates [58] that are eventually responsible for the slow kinetics of apophotoprotein activation. It should be noted that the pre-incubation with DTT has no noticeable effect on the rate of apo-obelin activation.

In effect, the formation of 2-hydroperoxycoelenterazine might be considered as a chemical reaction of coelenterazine with molecular oxygen occurring in a protein environment. The apparent rate of active photoprotein complex formation and consequently the apparent rate of coelenterazine conversion into peroxy adduct can be monitored by the increase of bioluminescence activity of apo-obelin mixed with coelenterazine in the activation buffer (Fig. 3). For wild type apo-obelin the apparent rate constant was evaluated to be $3.17 \pm 0.11 \times 10^{-3}$ s^{-1} at 20 °C and pH 6.5. Although the H175N and Y190F mutants revealed apparent dissociation constants equaling that of wild type apo-obelin the apparent rates of active photoprotein complex formation for these mutants were around one order of magnitude slower (Table 1). A similar decrease in the apparent rate of active apophotoprotein complex formation was observed for Y138F, Y138W and W179F (Table 1). Noteworthy is that although Y138W revealed the highest apparent dissociation constant indicating the lowest affinity to coelenterazine, the apparent rate of active photoprotein complex formation was found to be the largest among the studied group (Table 1).

4. Discussion

The side chains of His175, Trp179, and Tyr190 of photoprotein obelin (Fig. 1) are essential for stabilizing the 2-hydroperoxy adduct of coelenterazine and the subsequent Ca^{2+}-regulated light emitting reaction [49]. Tyr138 is another obelin residue involved in 2-hydroperoxycoelenterazine binding (Fig. 1). Substitution of Tyr138 to Phe causes a small long-wavelength shift in biolumines-

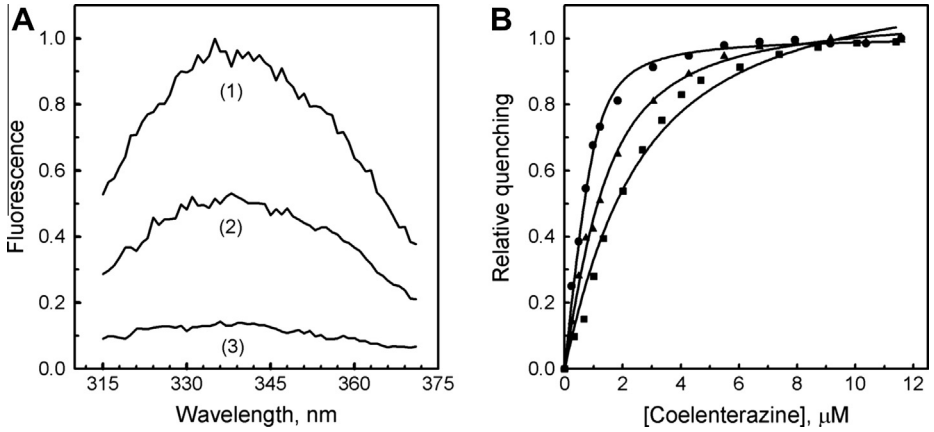


Fig. 2. Quenching of intrinsic apophotoprotein fluorescence by coelenterazine. (A) Scans displaying the effect of coelenterazine on apo-Y138F fluorescence measured between 315 and 375 nm (a) in absence of coelenterazine; (b) and (c) with 1.22 and 9.15 μM of coelenterazine, respectively. Spectra are normalized to the maximum of fluorescence without coelenterazine addition. (B) Determination of the apparent dissociation constants of wild type apo-obelin- (●), apo-Y138F- (▲), and apo-Y138 W-coelenterazine (■) complexes using the quenching of intrinsic fluorescence of apophotoproteins upon coelenterazine binding. The concentrations of apophotoproteins were 1.22 μM. All titrations were performed in triplicate.

Table 1
Bioluminescence activity, apparent dissociation constants and apparent rate constants of active photoprotein formation of obelin and its mutants.

	Bioluminescence activity (%)	Apparent dissociation constant K_D, (μM)	Apparent rate constant k, $\times 10^{-3}$ s^{-1}
Obelin WT	100.0	0.2 ± 0.04	3.17 ± 0.11
Y138F	60.0	0.65 ± 0.06	0.23 ± 0.02
Y138W	57.0	1.81 ± 0.2	0.87 ± 0.07
H175N	1.7	0.15 ± 0.01	0.37 ± 0.03
W179F	67.0	1.2 ± 0.07	0.43 ± 0.05
Y190F	14.3	0.2 ± 0.01	0.11 ± 0.01

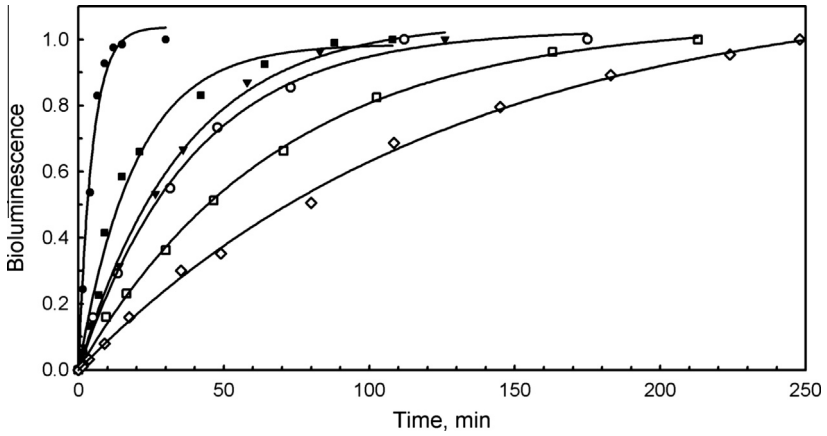


Fig. 3. Kinetics of active photoprotein complex formation from apophotoprotein, coelenterazine, and molecular oxygen monitored by bioluminescence for monomeric wild type apo-obelin (●), apo-Y138F (□), apo-Y138W (■), apo-H175H (○), apo-W179F (▼) and apo-Y190F (◇). Each kinetic curve is normalized to its respective maximum of bioluminescence activity. Apophotoprotein and coelenterazine concentrations were 50 and 750 nM respectively. The activation experiments were carried out at 20 °C and pH 6.5. All kinetic determinations were performed in triplicate. Apparent rate constants were calculated by one-exponential fitting of kinetic curves.

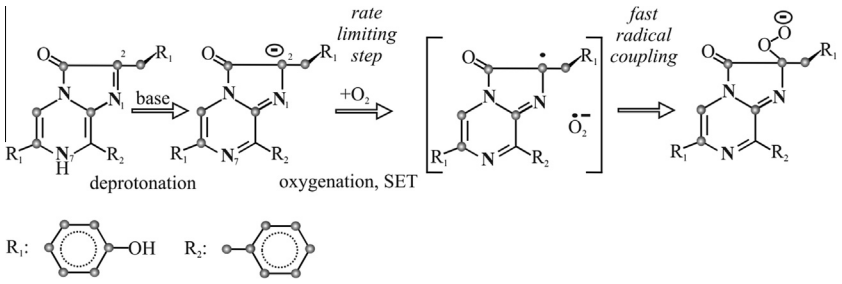
cence maximum but has no significant impact on bioluminescent activity [38]. To further our insight into initial active photoprotein complex formation from apophotoprotein, coelenterazine, and molecular oxygen, we studied selected Tyr138, His175, Trp179, and Tyr190 mutants regarding their capacity for coelenterazine binding and conversion into 2-hydroperoxy adduct.

The apparent dissociation constants for H175N and Y190F mutants were found to be equal to that of wild type obelin. In contrast, the apparent rate constants of active photoprotein complex formation for these mutants are almost 10 and 30 times lower (Table 1). This clearly shows that these residues are less critical for substrate binding but strictly important for catalyzing the conversion of coelenterazine into its 2-hydroperoxy derivative. Based on spectroscopic studies of the anaerobic complex of apo-obelin with coelenterazine [59] and molecular mechanisms suggested for several cofactor-independent oxidases and oxygenases according to which an active site histidine is involved in substrate activation with subsequent insertion of oxygen [60], we proposed that His175 in obelin might function as a proton shuttle in the process of active photoprotein complex formation from apophotoprotein, coelenterazine, and molecular oxygen. Since the H175N substitution slows down the rate of formation of active photoprotein complex we can reasonably conclude that this histidine indeed assists in the oxygenation of coelenterazine and might perform the role of proton shuttle in this process in the same manner as it occurs in case of cofactor-independent oxidases and oxygenases [60]. Since the Y190F substitution also reduces the rate of active photoprotein complex formation but does not influence the affinity to coelenterazine and because the side chains of His175 and Tyr190 might form hydrogen bonds with each other and with coelenterazine (Fig. 1) we can also suggest that both residues assist to (or participate directly in) the oxygenation of coelenterazine into 2-hydroperoxycoelenterazine.

Although Tyr138 is hydrogen bonded to the N1 atom of 2-hydroperoxycoelenterazine (Fig. 1) this residue is not essential for the bioluminescence because its replacement to Phe does not eliminate bioluminescence activity; Y138F obelin mutant preserves about 60% of bioluminescence activity of wild type obelin [38]. However the Y138F substitution clearly influences both the affinity of apophotoprotein for coelenterazine and the rate of coelenterazine conversion into 2-hydroperoxy adduct. The apparent dissociation constant of the apo-Y138F-coelenterazine complex is almost 3 times higher and the apparent rate constant of active photoprotein complex formation is nearly 14 times lower than that of wild type obelin (Table 1). For Y138W coelenterazine binding is one order of magnitude weaker than for wild type obelin, indicating that appearance of the bulky Trp138 side chain within the substrate binding cavity might create a steric hindrance for effective coelenterazine binding. The rate of active photoprotein complex formation for Y138W is only ~3.5 times lower than that of wild type obelin (Table 1). In case of Y138W, the amide function of the indole ring possibly forms a hydrogen bond with the N1 atom of coelenterazine thereby mimicking the hydrogen bond formed by the hydroxyl group of Tyr138 in wild type obelin (Fig. 1). This particular hydrogen bond is evidently missed in Y138F, allowing the conclusion that the capacity of Tyr138 to form this bond plays an important role in coelenterazine activation.

The apparent dissociation and rate constants for W179F are 6 times higher and 7 times lower as compared to those of wild type obelin (Table 1). Decreasing the dimensions of the hydrophobic side chain at position 179 influences the geometry and polarity of the substrate-binding cavity leading to a lower affinity for coelenterazine. In obelin, the nitrogen atom of the Trp179 ring is hydrogen bonded with the carbonyl oxygen of 2-hydroperoxycoelenterazine (Fig. 1). It is obvious that absence of this hydrogen bond in W179F decreases the rate of active photoprotein complex formation.

Studies of the chemiluminescence reaction of imidazopyrazinones with O_2 in aprotic solvents containing base have given a plausible mechanism for the formation of peroxy derivatives of



Scheme 2.

imidazopyrazinones [61–63]. According to the suggested reaction mechanism (Scheme 2), the first step is deprotonation of the N7 of imidazopyrazinone with a base yielding the imidazopyrazinone anion. Then, single electron transfer (SET) from imidazopyrazinone anion to triplet oxygen affords the imidazopyrazinone radical and the superoxide anion of oxygen ($O_2^{\bullet -}$). The SET process requiring spin conversion is considered to be rate-limiting. The radical coupling of imidazopyrazinone radical and superoxide anion leads to the peroxide anion of imidazopyrazinone. In case of bioluminescent reactions catalyzed by luciferases, the cyclization of imidazopyrazinone peroxide anion then will take place. The cyclization gives dioxetanone which decomposes with loss of CO_2 generating the singlet excited state of a product [63].

The mechanism of 2-hydroperoxycoelenterazine formation during conversion of apophotoprotein into photoprotein, in essence, might be the same with the only distinction: the coelenterazine peroxide anion resulting from radical coupling must be quickly protonated to prevent cyclization into dioxetanone [64]. For instance, a similar protonation of a peroxy adduct is operative in flavin-dependent aromatic hydroxylases [65].

The reaction of the formation of 2-hydroperoxy adduct of coelenterazine occurs within the substrate-binding cavity of apophotoprotein in an environment of amino acid residues which can form hydrogen bonds with coelenterazine atoms. It is evidently that these hydrogen bonds might influence the rates of individual reaction steps. Because it was proposed that SET is the rate-limiting step in the conversion of coelenterazine into peroxy anion (Scheme 2) [63] we can reasonably assume that in first instance the hydrogen bonds formed by Tyr138, His175, Trp179, and Tyr190 affect the single electron transfer step making this step more effective when all essential hydrogen bonds are present. However the force of their influence differs because, for example, the replacement of Tyr residues reduces the rates of active photoprotein complex formation to a greater extent than substitution of Trp and His. It should be noted also that His175 might affect not only the SET step. Since the substitution of this residue leads to significant loss of bioluminescence activity we might suppose that His can perform a dual function. The first one is a function of a "base" in subtracting a proton from coelenterazine with subsequent transfer to the peroxide anion (Scheme 2). The other function might be the influence through hydrogen bonding with either coelenterazine or Tyr190 on the SET process and consequently on the rate of active photoprotein complex formation. Since His175 is a strictly conserved residue the mechanism outlined above is supposed to be applicable for all hydromedusan Ca^{2+}-regulated photoproteins: aequorin, clytin and mitrocomin.

In summary, in the present work we have addressed the role of Tyr138, His175, Trp179 and Tyr190 in the active photoprotein complex formation process of the Ca^{2+}-regulated photoprotein obelin using a site-directed mutagenesis approach. We demonstrate that these four amino acid residues of the substrate-binding cavity are clearly important for active photoprotein complex formation because substitution of any of these residues significantly reduces the rate of coelenterazine conversion into its 2-hydroperoxy adduct.

Acknowledgements

The work was supported by RFBR grant 12-04-00131, by the Programs of the Government of Russian Federation "Measures to Attract Leading Scientists to Russian Educational Institutions" (grant 11.G34.31.0058), "Molecular and Cellular Biology" of RAS, President of Russian Federation "Leading science school" (grant 3951.2012.4). E.V.E. was supported by Wageningen University Sandwich PhD-Fellowship Program.

References

[1] S.H. Haddock, M.A. Moline, J.F. Case, Bioluminescence in the sea, Ann. Rev. Mar. Sci. 2 (2010) 443–493.
[2] E.A. Widder, Bioluminescence in the ocean: origins of biological, chemical, and ecological diversity, Science 328 (2010) 704–708.
[3] J.G. Morin, Coelenterate Bioluminescence, in: L. Muscatine, H.M. Lenhoff (Eds.), Coelenterate Biology: Reviews and New Perspectives, Academic Press, New York, 1974, pp. 397–438.
[4] C.M. Thomson, P.J. Herring, A.K. Campbell, The widespread occurrence and tissue distribution of the imidazolopyrazine luciferins, J. Biolumin. Chemilumin. 12 (1997) 87–91.
[5] Y. Ohmiya, T. Hirano, Shining the light: The mechanism of the bioluminescence reaction of calcium-binding photoproteins, Chem. Biol. 3 (1996) 337–347.
[6] J.W. Hastings, Chemistries and colors of bioluminescent reactions: a review, Gene 173 (1996) 5–11.
[7] O. Shimomura, F.H. Johnson, Structure of the light-emitting moiety of aequorin, Biochemistry 11 (1972) 1602–1608.
[8] M.J. Cormier, K. Hori, Y.D. Karkhanis, J.M. Anderson, J.M. Wampler, J.G. Morin, J.W. Hastings, Evidence for similar biochemical requirements for bioluminescence among the coelenterates, J. Cell. Physiol. 81 (1973) 291–297.
[9] E.S. Vysotski, J. Lee, Ca^{2+}-regulated photoproteins: structural insight into the bioluminescence mechanism, Acc. Chem. Res. 37 (2004) 405–415.
[10] O. Shimomura, Bioluminescence: chemical principles and methods, World Scientific, Singapore, 2006.
[11] D. Prasher, R.O. McCann, M.J. Cormier, Cloning and expression of the cDNA coding for aequorin, a bioluminescent calcium-binding protein, Biochem. Biophys. Res. Commun. 126 (1985) 1259–1268.
[12] S. Inouye, M. Noguchi, Y. Sakaki, Y. Takagi, T. Miyata, S. Iwanaga, T. Miyata, F.I. Tsuji, Cloning and sequence analysis of cDNA for the luminescent protein aequorin, Proc. Natl. Acad. Sci. USA 82 (1985) 3154–3158.
[13] D.C. Prasher, R.O. McCann, M. Longiaru, M.J. Cormier, Sequence comparisons of complementary DNAs encoding aequorin isotypes, Biochemistry 26 (1987) 1326–1332.
[14] T.F. Fagan, Y. Ohmiya, J.R. Blinks, S. Inouye, F.I. Tsuji, Cloning, expression and sequence analysis of cDNA for the Ca^{2+}-binding photoprotein, mitrocomin, FEBS Lett. 333 (1993) 301–305.
[15] S. Inouye, F.I. Tsuji, Cloning and sequence analysis of cDNA for the Ca^{2+}-activated photoprotein, clytin, FEBS Lett. 315 (1993) 343–346.
[16] S. Inouye, Cloning, expression, purification and characterization of an isotype of clytin, a calcium-binding photoprotein from the luminous hydromedusa *Clytia gregarium*, J. Biochem. 143 (2008) 711–717.
[17] S.V. Markova, L.P. Burakova, L.A. Frank, S. Golz, K.A. Korostileva, E.S. Vysotski, Green-fluorescent protein from the bioluminescent jellyfish *Clytia gregaria*: cDNA cloning, expression, and characterization of novel recombinant protein, Photochem. Photobiol. Sci. 9 (2010) 757–765.
[18] B.A. Illarionov, S.V. Markova, V.S. Bondar, E.S. Vysotski, I.I. Gitelzon, Cloning end expression of cDNA for obelin, calcium-activated photoprotein from the hydroid *Obelia longissima*, Dokl. Akad. Nauk. 326 (1992) 911–913.

[19] B.A. Illarionov, V.S. Bondar, V.A. Illarionova, E.S. Vysotski, Sequence of the cDNA encoding the Ca^{2+}-activated photoprotein obelin from the hydroid polyp *Obelia longissima*, Gene 153 (1995) 273–274.

[20] S.V. Markova, E.S. Vysotski, J.R. Blinks, L.P. Burakova, B.C. Wang, J. Lee, Obelin from the bioluminescent marine hydroid *Obelia geniculata*: cloning, expression, and comparison of some properties with those of other Ca^{2+}-regulated photoproteins, Biochemistry 41 (2002) 2227–2236.

[21] S.V. Markova, L.P. Burakova, S. Golz, N.P. Malikova, L.A. Frank, E.S. Vysotski, The light-sensitive photoprotein berovin from the bioluminescent ctenophore *Beroe abyssicola*: a novel type of Ca^{2+}-regulated photoprotein, FEBS J. 279 (2012) 856–870.

[22] M.R. Aghamaali, V. Jafarian, R. Sariri, M. Molakarimi, B. Rasti, M. Taghdir, R.H. Sajedi, S. Hosseinkhani, Cloning, sequencing, expression and structural investigation of mnemiopsin from *Mnemiopsis leidyi*: an attempt toward understanding Ca^{2+}-regulated photoproteins, Protein J. 30 (2011) 566–574.

[23] C.E. Schnitzler, K. Pang, M.L. Powers, A.M. Reitzel, J.F. Ryan, D. Simmons, T. Tada, M. Park, J. Gupta, S.Y. Brooks, R.W. Blakesley, S. Yokoyama, S.H. Haddock, M.Q. Martindale, A.D. Baxevanis, Genomic organization, evolution, and expression of photoprotein and opsin genes in *Mnemiopsis leidyi*: a new view of ctenophore photocytes, BMC Biol. 10 (2012) 107.

[24] M.L. Powers, A.G. McDermott, N.C. Shaner, S.H. Haddock, Expression and characterization of the calcium-activated photoprotein from the ctenophore *Bathocyroe fosteri*: Insights into light-sensitive photoproteins, Biochem. Biophys. Res. Commun. 431 (2013) 360–366.

[25] F.I. Tsuji, Y. Ohmiya, T.F. Fagan, H. Toh, S. Inouye, Molecular evolution of the Ca^{2+}-binding photoproteins of the Hydrozoa, Photochem. Photobiol. 62 (1995) 657–661.

[26] E.S. Vysotski, S.V. Markova, L.A. Frank, Calcium-regulated photoproteins of marine coelenterates, Mol. Biol. 40 (2006) 355–367.

[27] O. Shimomura, F.H. Johnson, Regeneration of the photoprotein aequorin, Nature 256 (1975) 236–238.

[28] J.R. Blinks, W.G. Wier, P. Hess, F.G. Prendergast, Measurement of Ca^{2+} concentrations in living cells, Prog. Biophys. Mol. Biol. 40 (1982) 1–114.

[29] J. Alvarez, M. Montero, Measuring $[Ca^{2+}]$ in the endoplasmic reticulum with aequorin, Cell Calcium 32 (2002) 251–260.

[30] T. Pozzan, R. Rudolf, Measurements of mitochondrial calcium *in vivo*, Biochim. Biophys. Acta 1787 (2009) 1317–1323.

[31] C. Grienberger, A. Konnerth, Imaging calcium in neurons, Neuron 73 (2012) 862–885.

[32] J.F. Head, S. Inouye, K. Teranishi, O. Shimomura, The crystal structure of the photoprotein aequorin at 2.3 Å resolution, Nature 405 (2000) 372–376.

[33] Z.J. Liu, E.S. Vysotski, C.J. Chen, J.P. Rose, J. Lee, B.C. Wang, Structure of the Ca^{2+}-regulated photoprotein obelin at 1.7 Å resolution determined directly from its sulfur substructure, Protein Sci. 11 (2000) 2085–2093.

[34] Z.J. Liu, E.S. Vysotski, L. Deng, J. Lee, J.P. Rose, B.C. Wang, Atomic resolution structure of obelin: soaking with calcium enhances electron density of the second oxygen atom substituted at the C2-position of coelenterazine, Biochem. Biophys. Res. Commun. 311 (2003) 433–439.

[35] M.S. Titushin, Y. Feng, G.A. Stepanyuk, Y. Li, S.V. Markova, S. Golz, B.C. Wang, J. Lee, J. Wang, E.S. Vysotski, Z.J. Liu, NMR-derived topology of a GFP-photoprotein energy transfer complex, J. Biol. Chem. 285 (2010) 40891–40900.

[36] L. Deng, S.V. Markova, E.S. Vysotski, Z.J. Liu, J. Lee, J. Rose, B.C. Wang, Crystal structure of a Ca^{2+}-discharged photoprotein: implications for mechanisms of the calcium trigger and bioluminescence, J. Biol. Chem. 279 (2004) 33647–33652.

[37] L. Deng, E.S. Vysotski, S.V. Markova, Z.J. Liu, J. Lee, J. Rose, B.C. Wang, All three Ca^{2+}-binding loops of photoproteins bind calcium ions: the crystal structures of calcium-loaded apo-aequorin and apo-obelin, Protein Sci. 14 (2005) 663–675.

[38] Z.J. Liu, G.A. Stepanyuk, E.S. Vysotski, J. Lee, S.V. Markova, N.P. Malikova, B.C. Wang, Crystal structure of obelin after Ca^{2+}-triggered bioluminescence suggests neutral coelenteramide as the primary excited state, Proc. Natl. Acad. Sci. USA 103 (2006) 2570–2575.

[39] Y. Ohmiya, M. Ohashi, F.I. Tsuji, Two excited states in aequorin bioluminescence induced by tryptophan modification, FEBS Lett. 301 (1992) 197–201.

[40] Y. Ohmiya, F.I. Tsuji, Bioluminescence of the Ca^{2+}-binding photoprotein, aequorin, after histidine modification, FEBS Lett. 320 (1993) 267–270.

[41] L. Deng, E.S. Vysotski, Z.J. Liu, S.V. Markova, N.P. Malikova, J. Lee, J. Rose, B.C. Wang, Structural basis for the emission of violet bioluminescence from a W92F obelin mutant, FEBS Lett. 506 (2001) 281–285.

[42] E.S. Vysotski, Z.J. Liu, S.V. Markova, J.R. Blinks, L. Deng, L.A. Frank, M. Herko, N.P. Malikova, J.P. Rose, B.C. Wang, J. Lee, Violet bioluminescence and fast kinetics from W92F obelin: structure-based proposals for the bioluminescence triggering and the identification of the emitting species, Biochemistry 42 (2003) 6013–6024.

[43] E.S. Vysotski, J. Lee, Bioluminescent mechanism of Ca^{2+}-regulated photoproteins from three-dimensional structures, in: V.R. Viviani, Y. Ohmiya (Eds.), Luciferases and fluorescent proteins: Principles and Advances in Biotechnology and Bioimaging, Transworld Research Network, Kerala, India, 2007, pp. 19–41.

[44] S. Inouye, Y. Sakaki, Y. Goto, F.H. Tsuji, Expression of apoaequorin complementary DNA in *Escherichia coli*, Biochemistry 25 (1986) 8425–8429.

[45] O. Shimomura, Y. Kishi, S. Inouye, The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues, Biochem. J. 296 (1993) 549–551.

[46] M.R. Knight, N.D. Read, A.K. Campbell, A.J. Trewavas, Imaging calcium dynamics in living plants using semi-synthetic recombinant aequorins, J. Cell. Biol. 121 (1993) 83–90.

[47] Y. Ohmiya, S. Kurono, M. Ohashi, T.F. Fagan, F.I. Tsuji, Mass spectrometric evidence for a disulfide bond in aequorin regeneration, FEBS Lett. 332 (1993) 226–228.

[48] E.V. Eremeeva, S.V. Markova, A.H. Westphal, A.J.W.G. Visser, W.J.H. van Berkel, E.S. Vysotski, The intrinsic fluorescence of apo-obelin and apo-aequorin and use of its quenching to characterize coelenterazine binding, FEBS Lett. 583 (2009) 1939–1944.

[49] E.V. Eremeeva, S.V. Markova, L.A. Frank, A.J.W.G. Visser, W.J.H. van Berkel, E.S. Vysotski, Bioluminescent and spectroscopic properties of His-Trp-Tyr triad mutants of obelin and aequorin, Photochem. Photobiol. Sci. 12 (2013) 1016–1024.

[50] W.L. DeLano, The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, 2002.

[51] S.V. Markova, E.S. Vysotski, J. Lee, Obelin hyperexpression in *E. coli*, purification and characterization, in: J.F. Case, P.J. Herring, B.H. Robison, S.H.D. Haddock, L.J. Kricka, P.E. Stanley (Eds.), Bioluminescence and Chemiluminescence, World Scientific Publishing Co., Singapore, 2001, pp. 115–119.

[52] B.A. Illarionov, L.A. Frank, V.A. Illarionova, V.S. Bondar, E.S. Vysotski, J.R. Blinks, Recombinant obelin: cloning and expression of cDNA purification, and characterization as a calcium indicator, Methods Enzymol. 305 (2000) 223–249.

[53] E.S. Vysotski, Z.J. Liu, J. Rose, B.C. Wang, J. Lee, Preparation and X-ray crystallographic analysis of recombinant obelin crystals diffracting to beyond 1.1 Å, Acta Crystallogr. D Biol. Crystallogr. 57 (2001) 1919–1921.

[54] H. Edelhoch, Spectroscopic determination of tryptophan and tyrosine in proteins, Biochemistry 6 (1967) 1948–1954.

[55] Y.J.M. Bollen, S.M. Nabuurs, W.J.H. van Berkel, C.P.M. van Mierlo, Last in, first out: the role of cofactor binding in flavodoxin folding, J. Biol. Chem. 280 (2005) 7836–7844.

[56] K. Kurose, S. Inouye, Y. Sakaki, F.I. Tsuji, Bioluminescence of the calcium-binding photoprotein aequorin after cysteine modification, Proc. Natl. Acad. Sci. USA 86 (1989) 80–84.

[57] J.C. Lewis, J.J. Lopez-Moya, S. Daunert, Bioluminescence and secondary structure properties of aequorin mutants produced for site-specific conjugation and immobilization, Bioconjugate Chem. 11 (2000) 65–70.

[58] H. Masuda, Y. Takenaka, Y. Shikamoto, M. Kagawa, H. Mizuno, F.I. Tsuji, Chromatography of isoforms of recombinant apoaequorin and method for the preparation of aequorin, Protein Exp. Purif. 31 (2003) 181–187.

[59] E.V. Eremeeva, P.V. Natashin, L. Song, Y. Zhou, W.J.H. van Berkel, Z.J. Liu, E.S. Vysotski, Oxygen activation of apo-obelin – coelenterazine complex, Chembiochem 14 (2013) 739–745.

[60] S. Fetzner, R.A. Steiner, Cofactor-independent oxidases and oxygenases, Appl. Microbiol. Biotechnol. 86 (2010) 791–804.

[61] T. Goto, Chemistry of bioluminescence, Pure Appl. Chem. 17 (1968) 421–441.

[62] T. Goto, S. Inoue, S. Sugiura, Cypridina bioluminescence. IV. Synthesis and chemiluminescence of 3,7-dihydroimidazo[1,2-a]pyrazin-3-one and its 2-methyl derivative, Tetrahedron Lett. 9 (1968) 3873–3876.

[63] H. Kondo, T. Igarashi, S. Maki, H. Niwa, H. Ikeda, T. Hirano, Substituent effects on the kinetics for the chemiluminescence reaction of 6-arylimidazo[1,2-a]pyrazin-3(7H)-ones (Cypridina luciferin analogues): support for the single electron transfer (SET)–oxygenation mechanism with triplet molecular oxygen, Tetrahedron Lett. 46 (2005) 770–7704.

[64] F.G. Prendergast, Bioluminescence illuminated, Nature 405 (2000) 291–293.

[65] B. Entsch, W.J.H. van Berkel, Structure and mechanism of para-hydroxybenzoate hydroxylase, FASEB J. 9 (1995) 476–483.