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FEBS Letters 583 (2009) 1939–1944

FEBS Letters
journal homepage: www.FEBSLetters.org

The intrinsic fluorescence of apo-obelin and apo-aequorin and use of its quenching to characterize coelenterazine binding

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ARTICLE INFO

Article history:
Received 24 March 2009
Revised 23 April 2009
Accepted 28 April 2009
Available online 6 May 2009

Edited by Richard Cogdell

Keywords:
Bioluminescence
Photoprotein
Trp fluorescence

ABSTRACT

The intrinsic fluorescence of two apo-photoproteins has been characterized and its concentration-dependent quenching by coelenterazine has been for the first time applied to determine the apparent dissociation constants for coelenterazine binding with apo-aequorin (1.2 ± 0.12 μM) and apo-obelin (0.2 ± 0.04 μM). Stopped-flow measurements of fluorescence quenching showed that coelenterazine binding is a millisecond-scale process, in contrast to the formation of an active photoprotein complex taking several hours. This finding evidently shows that the rate-limiting step of active photoprotein formation is the conversion of coelenterazine into its 2-hydroperoxy derivative.
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1. Introduction

The Ca^{2+}-regulated photoproteins consist of a single polypeptide chain of about 22 kDa to which the substrate, 2-hydroperoxy-coelenterazine, is tightly though not covalently bound [1]. Photoprotein bioluminescence is triggered by Ca^{2+} binding which induces the generation of protein-bound coelenteramide in its excited state. Because photoproteins are highly sensitive to detect calcium and harmless when injected into living cells, they have been widely used as probes of intracellular Ca^{2+}. The successful cloning of cDNAs encoding photoproteins has opened a new way of utilizing photoproteins, by expressing the recombinant apo-photoprotein intracellularly, then adding coelenterazine externally which diffuses into the cell and forms the active photoprotein [2,3]. This technique is highly valuable, because it does not require microinjection.

Our previous studies have focused on the mechanism of photoprotein bioluminescence [4]. At the same time much less is known about the mechanism of formation of the active photoprotein complex (Scheme 1); there are investigations on relative rates of the regeneration *in vitro* of wild-type aequorin with coelenterazine and its analogs as well as the effect of temperature, pH, incubation time, reducing agent concentrations, and some additives on this process [5–8]. It was also shown that DTT (or β-mercaptoethanol) is required to reduce disulfide bonds in the recombinant apo-aequorin [9].

All photoproteins with known amino acid sequence contain six tryptophans. Four residues (Trp92, 114, 135, and 179; numbered according to the obelin sequence [10]) are found in the coelenterazine-binding pocket (Fig. 1). Trp18 and Trp103 are situated away from the binding cavity in the first and fourth α-helix, respectively (Fig. 1C). The side chains of Trp92 and Trp179 sandwich the 6-(*p*-hydroxyphenyl) ring of coelenterazine; the side chains of Trp114 and Trp135 are localized near the 2-(*p*-hydroxybenzyl) group of coelenterazine (Fig. 1). The nitrogen atoms of the Trp92 and Trp179 indole rings interact dipolarly with the oxygen atoms of the 6-(*p*-hydroxyphenyl) group and the C3 carbonyl of coelenterazine [11]. It has been shown that Trp92 affects the bioluminescence emitter because its replacement with Phe or some other residue leads to a change in the light emission spectrum of photoproteins [12–15]. Five Trp residues occupy the same positions in obelin and aequorin. The Trp103 of obelin (Fig. 1A) and Trp78 of aequorin (Fig. 1B) are found in the fourth α-helix but at the opposite termini (Fig. 1) [10,16]. The spatial structures of different ligand-dependent conformational states of obelin show that only these tryptophans might be accessible to solvent [10,17–19]. It should be noted, however, that the spatial structure of apo-photoprotein is not yet known.

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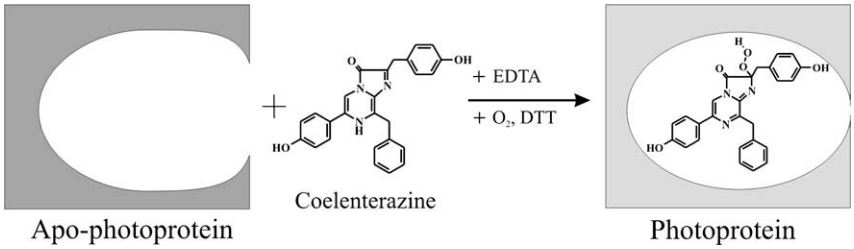
doi:10.1016/j.febslet.2009.04.043



Scheme 1.

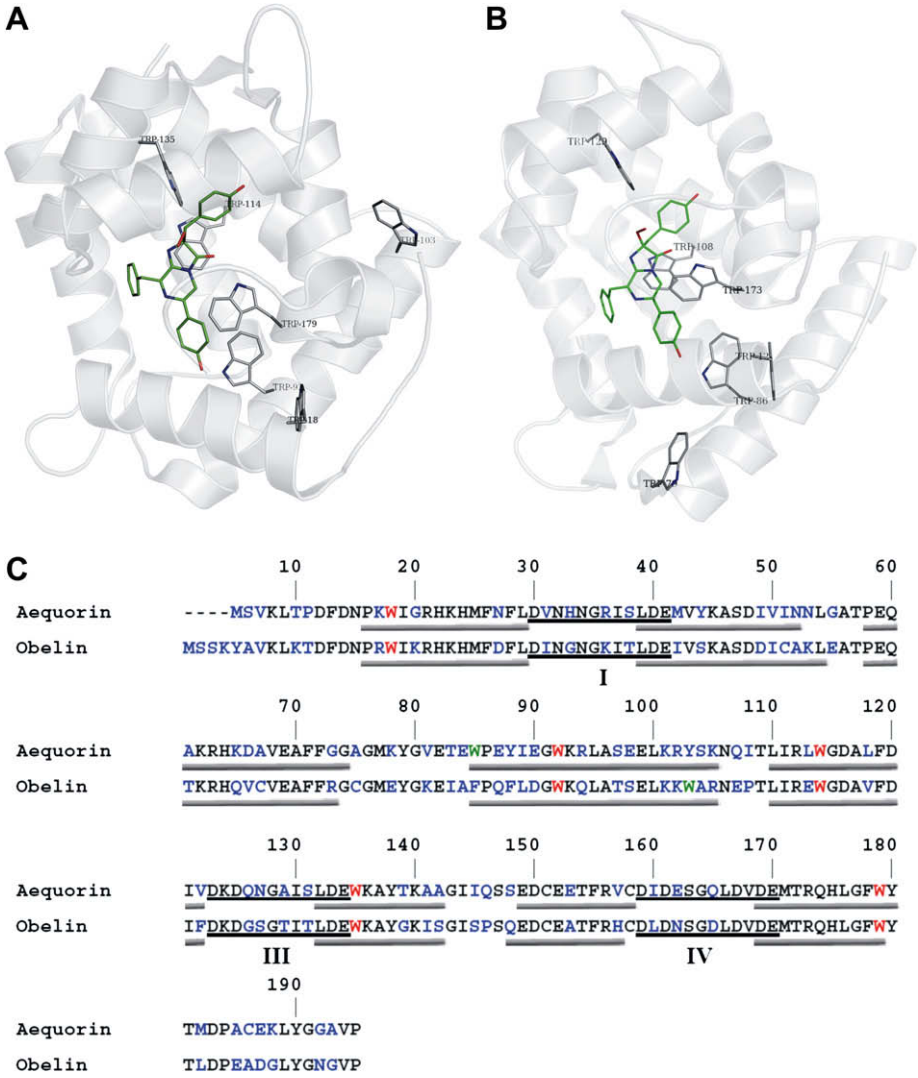


Fig. 1. Spatial structures of obelin (PDB code 1QV0) (A) and aequorin (PDB code 1EJ3) (B), and their sequence alignment (C). The residues of aequorin structure (B) are numbered according to 1EJ3. The 2-hydroperoxycoelenterazine molecule (green) is shown in the center as a stick model. The identical and variable residues are in black and blue letters, respectively. The conservative and non-conservative Trp residues are colored by red and green, respectively; Ca^{2+}-binding loops I, III, and IV are underlined. The gray cylinders show α-helices according to photoprotein structures.

In this paper we outline for the first time the intrinsic fluorescence of apo-obelin and apo-aequorin and use of its quenching for the characterization of coelenterazine binding.

2. Materials and methods

2.1. Site-directed mutagenesis

The site-directed mutagenesis was done on the template pET19-OL8 *Escherichia coli* expression plasmid carrying the *Obelia longissima* wild-type apo-obelin gene [20]. Mutations resulting in the changes – W18F, W92F, W103F, W114F, W135F, or W179F – were carried out with the QuickChange site-directed mutagenesis kit (Stratagene, USA) according to the protocol supplied with the kit. The plasmids harboring the mutations were verified by DNA sequencing.

2.2. Protein expression and purification

The apo-obelin and apo-aequorin truncated by six residues from the N-terminus as well as obelin mutants were expressed as previously reported [18,21]. For protein production, the trans-

formed *E. coli* BL21-Gold was cultivated with vigorous shaking at 37 °C in LB medium containing 200 μg/ml ampicillin and induced with 1 mM IPTG when the culture reached an OD600 of 0.5–0.6. After addition of IPTG, the cultivation was continued for 3 h. Most of the apo-photoproteins produced were accumulated in inclusion bodies.

Apo-obelin and apo-aequorin were purified from inclusion bodies as described [22]. The apo-photoproteins 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 apo-photoproteins, 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 apo-photoproteins (~1 mg/ml) were 0.5 ml-aliquoted into plastic tubes and frozen at −80 °C.

All the experiments with apo-photoproteins were carried out with freshly thawed samples. Thawed samples were centrifuged (20 000×*g* × 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 apo-photoprotein containing no disulfide bonds and aggregates [9,23]. The final preparations of apo-photoproteins were homogeneous according to SDS–PAGE and gel filtration. Obelin and aequorin, charged with coelenterazine, were produced as previously reported [21,22,24].

The coelenterazine concentration in the methanol stock solution was determined spectrophotometrically using the extinction coefficient (ε_{434nm} = 8900 cm^{-1} M^{-1} [25]). The apo-obelin and apo-aequorin concentrations were determined using ε_{280nm} = 40 450 and 43 430 M^{-1} cm^{-1}, respectively, as calculated with the ProtParam tool (http://us.expasy.org/tools/protparam-doc.html) which uses Edelhoch's method [26].

2.3. Fluorescence measurements

Fluorescence measurements were carried out with an AMINCO spectrofluorimeter (Thermo Spectronic, USA) in 5 mM EDTA, 10 mM DTT, 20 mM Tris–HCl pH 7.0 at 20 °C. Excitation was at 295 nm (slit 4 nm). The fluorescence emission spectra were corrected with the computer program supplied with the instrument. To assess fluorescence quenching only the changes of fluorescence intensity at 336 nm were used. All spectra were taken using a standard quartz cuvette (1 × 1 cm) in a 1-ml initial volume with varied coelenterazine additions in 1- to 5-μl portions up to saturation. 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:

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

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

2.4. Determination of apparent dissociation constant of the apo-photoprotein–coelenterazine complex

The apparent dissociation constant of the apo-photoprotein–coelenterazine complex was determined using the quenching of apo-obelin and apo-aequorin Trp fluorescence upon binding to coelenterazine. Our analysis assumed 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. (1), a modified equation compared with the one described elsewhere [27]:

$$\frac{Q}{Q_{max}} = \frac{(C + L + K_D) - \sqrt{(C + L + K_D)^2 - 4CL}}{2} \quad (1)$$

where C, L, and K_D are apo-photoprotein and coelenterazine concentrations, and apparent dissociation constant, respectively.

2.5. Stopped-flow measurements

Stopped-flow measurements were carried out using a temperature-controlled Hi-Tech SF-51 apparatus equipped with a Hi-Tech SU-40 spectrometer (dead time 1.5 ms) (Salisbury, United Kingdom) at 20 °C. The changes in apo-photoprotein fluorescence emission upon coelenterazine binding were detected between 300 and 400 nm (using Corning WG320 and UG380 filters) with excitation set at 280 nm. The spectral excitation bandwidth was 10 nm. The stopped-flow measurements were carried out by mixing of 1.2 μM apo-obelin or 1.2 μM apo-aequorin in 5 mM EDTA, 10 mM DTT, 20 mM Tris–HCl pH 7.0 with either 1.2 or 6.0 μM coelenterazine prepared in the same buffer.

3. Results and discussion

3.1. Intrinsic fluorescence of apo-obelin and apo-aequorin

Both apo-photoproteins have typical UV absorption properties characteristic for proteins without any organic ligand with a maximum absorbance at 280 nm and a shoulder at 295 nm (Fig. 2A). The active photoproteins, i.e. having bound 2-hydroperoxycoelenterazine, in addition display a shoulder at 310 nm and a maximum at 460 nm. Freshly prepared coelenterazine shows absorption maxima at 275 and 425 nm, and a shoulder at 340 nm.

With excitation at 295 nm, the intrinsic fluorescence of apo-photoproteins is observed with a maximum at 336 nm (Fig. 2B). Given that the excitation is at 295 nm, the observed fluorescence is due exclusively to Trp fluorescence. The photoprotein preparations, separated from uncharged apo-proteins by ion-exchange chromatography on Mono Q [21,24], display only a very weak fluorescence with an intensity of about 5–6% of apo-photoprotein fluorescence and with the same maximum (336 nm). Free coelenterazine does not display any fluorescence in the range from 310 to 400 nm; a very weak fluorescence is observed beginning at 400 nm with low intensity maxima at 440 and 520 nm (Fig. 2B). These results evidently show that coelenterazine binding to apo-photoproteins quench Trp fluorescence.

The very low fluorescence of active photoproteins is rather surprising because one of the Trp residues of obelin (Trp103) and aequorin (Trp79) (Fig. 1) is found on the surface of the protein and is accessible to solvent. Examination of the surroundings of these tryptophans in the crystal structures of obelin (PDB code 1QV0) and aequorin (PDB code 1EJ3) gives a possible explanation for the low intensity of the intrinsic fluorescence. Nearby the indole rings of Trp103 in obelin and Trp79 in aequorin are found the side chains of Arg173 and Lys17, respectively. Although in the crystal structures the atoms of these residues do not form elec-

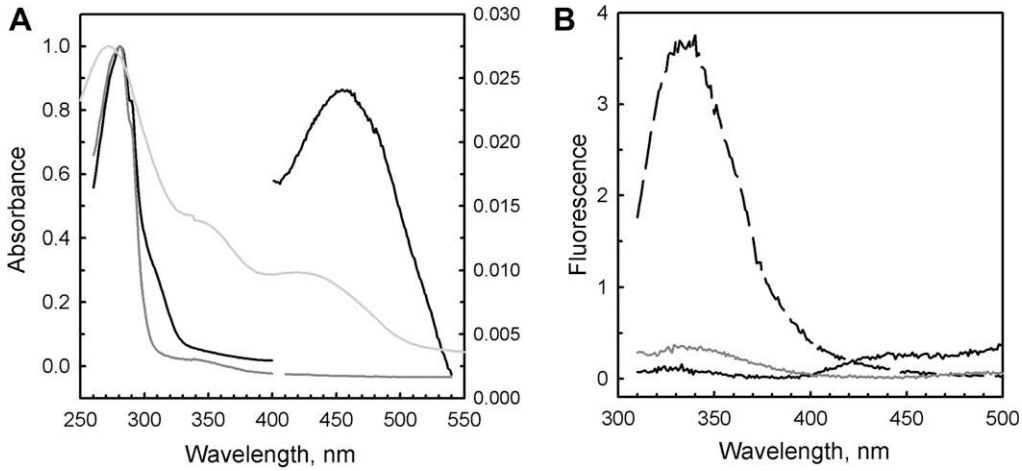


Fig. 2. Absorption and fluorescence properties of photoproteins. (A) Normalized absorption spectra of apo-aequorin (dark gray line), active aequorin (black line), and free coelenterazine (gray line). The right black and dark gray spectra (after break) belong to the right scale. (B) Fluorescence emission spectra of apo-obelin (dashed black line), active obelin (dark gray), and free coelenterazine (black line) with excitation at 295 nm. All samples were in 1 mM EDTA, 20 mM Tris–HCl pH 7.0. To minimize effect of autooxidation, the coelenterazine in buffer was prepared immediately before spectral measurements from methanol stock solution.

trostatic interactions with Trp atoms, dynamic interactions with these polar residues might be present in solution and effectively quench the fluorescence.

3.2. Intrinsic fluorescence of Trp mutants of obelin

To estimate the influence of each tryptophan on the intrinsic fluorescence properties of apo-photoprotein we produced six obelin mutants by replacing each Trp in turn by Phe (Fig. 3). The substitutions do not change the wavelength of the fluorescence emission maxima in comparison with those of wild-type apo-obelin but influence the fluorescence intensity. Although Trp92 deeply resides within the coelenterazine-binding pocket (Fig. 1), the decrease in relative intensity on its substitution amounts to ~40% of the total fluorescence signal. The substitution of the other two tryptophans of the coelenterazine-binding cavity, Trp114 and Trp179, as well as Trp18 situated in the first α-helix, results in a decrease of nearly 20% of the total fluorescence of apo-obelin. Substitution of Trp135, the fourth tryptophan of the coelenterazine-binding cavity, has practically no effect on the overall intensity, indicating that most probably the fluorescence from this Trp is severely quenched by interactions within the protein. Trp103 is the only tryptophan on the surface of the photoprotein molecule (Fig. 1). Its substitution reduces the intrinsic fluorescence approximately by 5%. It is quite possible that this Trp is responsible for the weak fluorescence of the active photoproteins (Fig. 2B). The different effects of Trp substitutions on the total intrinsic fluorescence of apo-obelin are likely due to different efficiencies of their fluorescence quenching which might depend on local environments of each Trp.

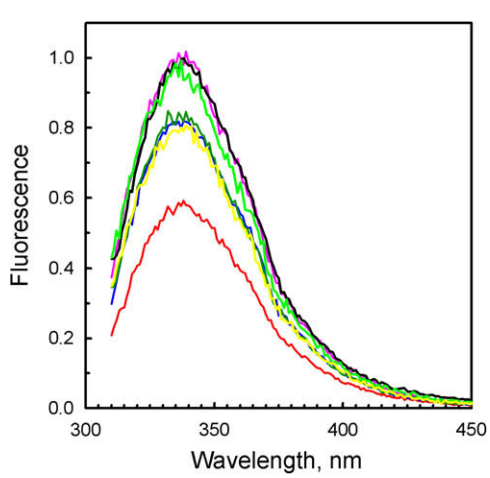


Fig. 3. Normalized fluorescence emission spectra of wild-type apo-obelin (black line) and its tryptophan mutants: W18F, yellow; W92F, red; W103F, light green; W114F, blue; W135F, pink; and W179F, dark green. The excitation wavelength is 295 nm. The concentrations of wild-type apo-obelin and its mutants are 1.22 μM.

3.3. Quenching of Trp fluorescence of apo-obelin and apo-aequorin with coelenterazine

Addition of coelenterazine to apo-obelin induces a strong concentration-dependent decrease of Trp fluorescence (Fig. 4A). Similar results were obtained with apo-aequorin (not shown). However, even a 7-fold molar excess of coelenterazine does not quench the intrinsic fluorescence of apo-photoproteins completely; always there is residual fluorescence constituting approximately 20–30% of the initial fluorescence. The residual fluorescence hardly changes; even after four hours (time required to form an active photoprotein) the fluorescence intensity is decreased by only 2–4%. In contrast, the intrinsic fluorescence of photoproteins after ion-exchange chromatography on Mono Q column is very weak (5–6% of apo-photoprotein intensity) (Fig. 2). This might indicate that approximately a quarter of apo-photoprotein is incompetent for coelenterazine binding despite the fact that monomeric apo-photoproteins reduced with DTT have been used. It is tempting to equate this quarter with the empirical observation that on charging the recombinant apo-photoprotein with coelenterazine, there is always about the same fraction of uncharged protein, which can be separated by ion-exchange chromatography [21,24]. It should be noted that the residual (20–30%) fluorescence has approximately the same maximum (336 nm) indicating that the Trp residues of binding-incompetent apo-photoprotein have a very similar environment.

The dissociation constant of the apo-photoprotein–coelenterazine complex was determined using the above quenching tryptophan fluorescence data by fitting the relative fluorescence emission to Eq. (1) (see "Section 2") (Fig. 4B). The estimated dissociation constants turn out to be 1.2 ± 0.12 and 0.2 ± 0.04 μM for apo-aequorin and apo-obelin, respectively, indicating that the affinity of apo-aequorin to coelenterazine is lower than that of apo-obelin.

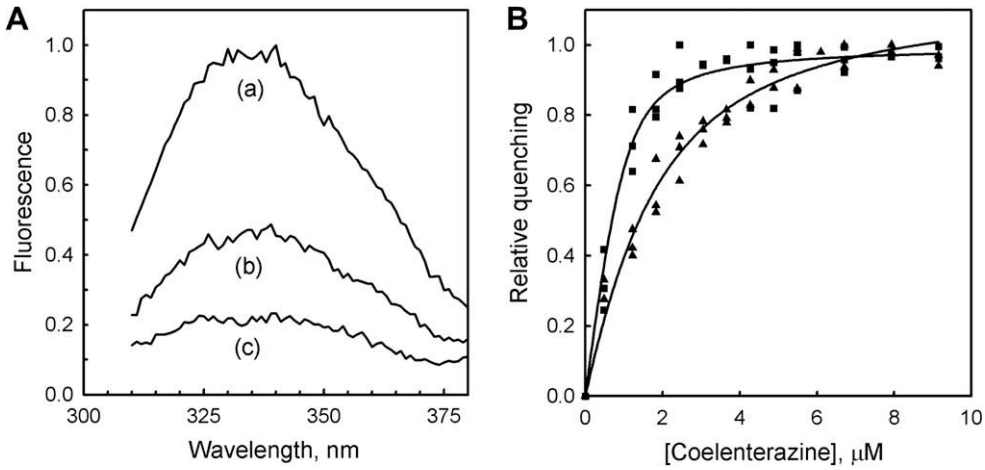


Fig. 4. Quenching of intrinsic apo-photoprotein fluorescence by coelenterazine. (A) Scans exhibiting the effect of coelenterazine on apo-obelin fluorescence measured between 310 and 380 nm: (a) in absence of coelenterazine; (b) and (c) with 1.22 and 9.15 μM of coelenterazine, respectively. (B) Determination of the apparent dissociation constants of apo-obelin- (■) and apo-aequorin-coelenterazine (▲) complexes using the quenching of intrinsic fluorescence of apo-photoproteins upon coelenterazine binding. Eq. (1) is fitted to the fluorescence intensity data as described under "Section 2". The concentrations of apo-obelin and apo-aequorin were 1.22 μM. All titrations were performed in triplicate.

This result is somewhat surprising because according to the spatial structures of these photoproteins the residues lining the coelenterazine-binding cavity are almost the same; there is a distinction in only three amino acids [10,16]. In obelin, Ile42, Ile50, and Phe88 occupy similar positions as Met42, Val50, and Tyr88 in aequorin (Fig. 1). Probably two of these residues (Met and Tyr) might affect aequorin affinity to coelenterazine because the properties of their side chains differ from those of Ile and Phe. This assumption seems quite reasonable because, for example, the substitution of Tyr88 in aequorin by Phe (or Phe88 in obelin by Tyr) leads to changes in bioluminescence and fluorescence maxima [28].

It should be noted that the determined dissociation constants for coelenterazine are not equilibrium dissociation constants in the strict sense. When the quenched apo-photoprotein (it was tested with apo-obelin and apo-aequorin) was diluted by a factor of two immediately after coelenterazine addition an exactly 2-fold decrease of fluorescence was observed that obviously shows that coelenterazine remains tightly associated with apo-photoprotein. Most likely the strong association of coelenterazine with apo-photoprotein results from hydrophobic interactions of coelenterazine with non-polar residues lining the coelenterazine-binding cavity placing this substrate in proximity to Trp residues. Hence it is more appropriate to use the term "apparent dissociation constant".

A methanol solution of coelenterazine displays weak yellow fluorescence [1]. A similar weak fluorescence is observed with freshly prepared coelenterazine in buffer (Fig. 5). However, the fluorescence intensity of coelenterazine is noticeably increased upon binding to apo-photoprotein and is accompanied with a blue shift in emission. Because the absorption spectrum of coelenterazine in buffer has a shoulder at 340 nm (Fig. 2A) corresponding to the tryptophan fluorescence maximum of apo-photoproteins we might reasonably assume that the increase of coelenterazine fluorescence results from resonance energy transfer between tryptophans and bound coelenterazine. Since the fluorescence of coelenterazine is very weak we might also surmise it is RET that results in the quenching of apo-photoprotein intrinsic fluorescence by coelenterazine.

The quenching of intrinsic fluorescence by coelenterazine and therefore coelenterazine binding with apo-photoprotein is very fast because the Trp fluorescence of apo-obelin and apo-aequorin measured right after adding a 5-fold molar excess of coelenterazine is immediately quenched and no further significant change of fluorescence is observed. Experiments using the stopped-flow technique showed that coelenterazine binding takes place within the dead-time (1.5 ms) of the instrument (not shown). Thus, we can conclude that the coelenterazine binding with apo-photoproteins is a very fast process occurring on submillisecond time scale.

In conclusion, in this paper for the first time we have characterized the intrinsic fluorescence of two apo-photoproteins and demonstrated that the quenching of Trp fluorescence by coelenterazine can be applied for studying its binding with apo-protein. Using the effect of concentration-dependent quenching of the intrinsic fluorescence of apo-photoproteins upon binding of coelenterazine we have determined the apparent dissociation constants for coelenterazine binding with apo-aequorin and apo-obelin. The values of apparent dissociation constants imply a high affinity of the apo-photoprotein substrate-binding cavity for coelenterazine. The binding of coelenterazine is shown to be a fast process taking less than milliseconds. This indicates that the rate-limiting step of active photoprotein complex formation is the relatively slow conversion of coelenterazine into its peroxy adduct.

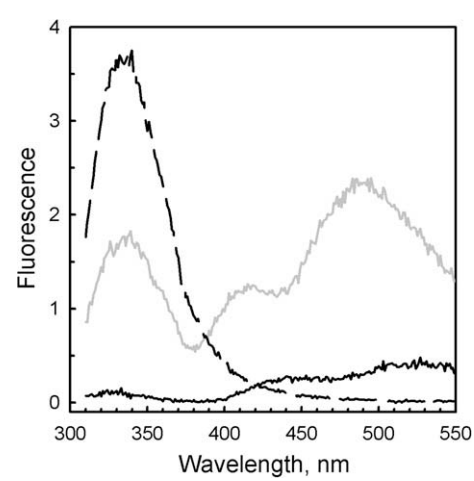


Fig. 5. Fluorescence emission spectra of 1.22 μM apo-obelin (dashed black line), freshly prepared 1.22 μM coelenterazine in the same buffer (solid black line) and their mixture at equimolar concentrations of 1.22 μM (gray line). Excitation wavelength is 295 nm.

Acknowledgments

We thank Prof. John Lee for valuable suggestions and providing constructive criticisms. The work was supported by Wageningen University Sandwich PhD-Fellowship Program, Grants 02.512.12. 2006 and 1211.2008.4 of Ministry of Education and Science of Russian Federation, MCB Program of RAS, and by Grant No. 2 of SB RAS.

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