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Structural basis for the emission of violet bioluminescence from a W92F obelin mutant

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Abstract Mutation of the Trp92 that is known to lie within the active site of the photoprotein obelin from *Obelia longissima*, results in a shift of the bioluminescence color from blue (λ_{max} = 485 nm) to violet. The corrected spectrum shows a new band with λ_{max} = 410 nm now contributing equally to the one at longer wavelength. The crystal structure of this W92F obelin determined at 1.72 Å resolution shows that there is no significant change in the dimensions of the active site between WT obelin (recombinant Ca^{2+}-regulated photoprotein from *Obelia longissima*) and the mutant. It is proposed that the bioluminescence spectral shift results from removal of a hydrogen bond from the indole of W92 nearby a hydroxyl belonging to the 6-phenyl substituent of the substrate coelenterazine. Propagation of this change through a conjugated bond system in the excited state of the product coelenteramide affects the coupling of the N1-position and the hydrogen-bonded Y138. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Calcium-regulated photoprotein; X-ray crystallography; Fluorescence; Coelenterazine; Aequorin

1. Introduction

Calcium-regulated photoproteins are found in the marine bioluminescent coelenterates [1], the most well-known being Aequorin, from the bioluminescent jellyfish Aequorea [2]. They are named because it only requires the addition of Ca^{2+} to trigger the light reaction, with the emission of bioluminescence of blue to blue–green color. Their bioluminescence spectral distributions are broad with maxima depending on the species in the range 465–495 nm. It had been proposed that the lack of any substrate requirement for the light reaction is because the protein contains an imidazopyrazinone derivative named 'coelenterazine' tightly bound within the protein's active site and as a stabilized peroxide derivative (**I**). Many chemical and biochemical studies have established the reaction to be an oxidative decarboxylation of this peroxycoelenterazine with the exergonicity deposited into the first electronic state of the product, coelenteramide (Scheme 1).

Fluorescence studies of coelenteramide and analogues have shown that several ionic species can exist, a neutral species (**II**) with fluorescence spectral maximum around 400 nm, the amide monoanion (**III**) around 450 nm, a phenolate anion (**IV**) 480–490 nm, and other anionic species with maxima at longer wavelengths [3]. Small variations are also introduced by solvent dielectric constant and solvent viscosity changes. The general consensus is that the excited state **III** is the source of the bioluminescence spectrum but in obelin (λ_{max} = 485 nm) there is a small band at 400 nm [4] evidently from the excited **II** [3]. Part of the identifying evidence for **III** as the primary excited state therefore was in the case of aequorin, a close match between its bioluminescence spectrum (λ_{max} = 465 nm) and the fluorescence of the protein-bound product. However for other photoproteins such as obelin, the product fluorescence is not the same and is at longer wavelength than the bioluminescence [4]. It is suggested that the Ca^{2+}-discharged obelin fluorescence is from **IV**, as it is matched by the fluorescence of coelenteramide in certain basic apolar solvents [3].

Very recently the three-dimensional structures of two recombinant photoproteins have been determined, aequorin at resolution 2.3 Å [5], and obelin from the hydroid *Obelia longissima*, at resolutions 1.7 [6] and 1.1 Å [7]. Both photoproteins exhibit spatial structure characteristics of the super-family of Ca^{2+}-regulated proteins, four helix–turn–helix motifs, but only three motifs are Ca^{2+}-binding EF-hands. Of considerable importance for mechanistic study is that these structures show the substrate stabilized within the active site. Both protein structures indicate that the coelenterazine is substituted at the C2-position. In the aequorin crystal, weak electron density at this C2-position is consistent with the presence of a peroxy group but there is clearly only one oxygen atom in both obelin structures. It has been suggested that the latter represents the substrate prior to oxygen addition, perhaps bound as a hydrate [7].

Ohmiya et al. [8] produced a number of Trp to Phe mutants of aequorin and found that one of them, W86F, showed a bioluminescence emission with the 400-nm band enhanced, i.e. a contribution to the emission from **II**. We show here that the equivalent mutation in obelin, W92F, produces an even greater enhancement and that there is no significant change in the

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Abbreviations: WT obelin, recombinant Ca^{2+}-regulated photoprotein from *Obelia longissima*

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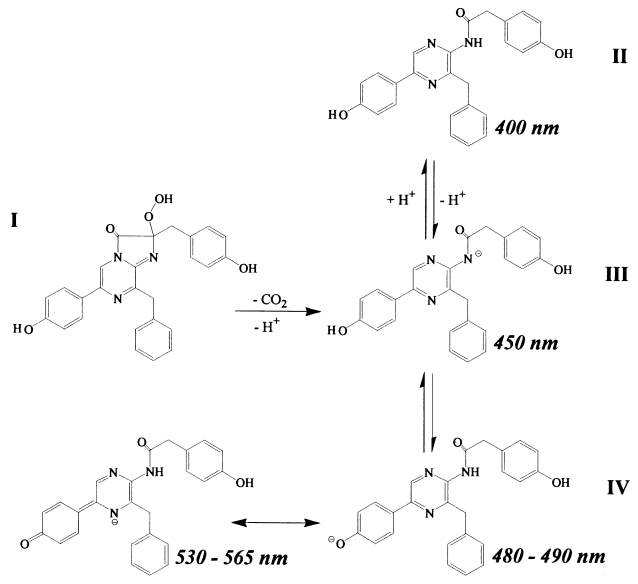

Scheme 1.

dimensions of the active site between WT obelin and the mutant. The spatial structure now provides a rational basis for explaining the enhancement of the 400-nm bioluminescence. It is proposed that the bioluminescence spectral shift results from removal of the influence of hydrogen bonding from the indole of W92 which lies nearby the hydroxyl belonging to the 6-phenyl substituent of the substrate coelenterazine.

2. Materials and methods

2.1. Molecular biology

Site-directed mutagenesis was done on the template pET19-OL8 *Escherichia coli* expression plasmid carrying the *O. longissima* wild-type apo-obelin (herein called WT obelin) [4]. Mutation resulting in the amino acid change W92F was introduced using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. Two complementary oligonucleotides containing the desired mutations were the sense primer 5′-CAATTCCTCGATGGATTCAAACAATTAGCG-3′ and antisense primer 5′-CGCCAATTGTTTGAATCCATCGAGGAATTG-3′. The changed nucleotides causing the mutations are underlined. Plasmid harboring the mutation was verified by DNA sequence analysis.

For protein production, the *E. coli* BL21-Gold cells with the W92F-obelin plasmid were cultivated with vigorous shaking at 37°C in LB medium containing ampicillin and induced with 1 mM isopropyl β-D-thiogalactopyranoside when the culture reached an OD_{600} of 0.5–0.6. After induction, the cultivation was continued for 3 h.

2.2. Protein purification, crystallization and data collection

W92F obelin was purified as previously reported [4,9]. The purified protein was concentrated to approximately 8–10 mg/ml, desalted on a BioGel P2 column equilibrated with 1 mM EDTA, 10 mM Na/K phosphate buffer, pH 7.3, and concentrated again to approximately the same concentration. Protein was homogeneous according to LC-Electrospray Ionization Mass Spectrometry and the mass was in excellent agreement with that calculated from sequence excluding Met1, as also observed for WT obelin [10]. Protein concentration was determined with the Bio-Rad DC Protein Assay Kit with bovine serum albumin as a protein standard.

W92F obelin was crystallized under conditions similar to WT obelin [7] by the hanging drop vapor diffusion technique at 4°C. The best precipitant was a solution containing 18% polyethylene glycol 8000, 50 mM KH_2PO_4, pH 6.0, with the addition of hexaminecobalt chloride into the drop. Obelin crystals had a size of about $0.5\times0.25\times0.25$ mm after 10–15 days. Crystals exposed to calcium ion before and after X-ray irradiation, emit light confirming that the crystals consist of an active photoprotein. A complete diffraction data set to 1.72 Å resolution has been collected at 100 K, on a RAXIS IV image plate detector using MSC blue osmic confocal optics focused 5.0 kW Cu K_α X-rays generated on a Rigaku RU200 rotating anode. The data were indexed, integrated, and scaled using DENZO/SCALEPACK.

2.3. Refinement

The structure was determined by the Molecular Replacement Method using WT obelin (protein data base entry: 1EL4) as a search model. The space group is $P4_12_12$ with unit-cell dimensions $a = b = 53.45$ Å and $c = 144.49$ Å. The refinement was carried out using CNS1.0 [11]. The final R-value is 23.4%. The free R-factor value is 27.6% using 8% reflection test set. The root-mean-square deviation (r.m.s.d.) in bond length and angles are 0.005 Å and 1.1° respectively. Analysis of the Ramachandran plot (PROCHECK) [12] showed that 94% residues are in most favored regions and 6% residues are in additional allowed regions. No residues are in disallowed or generously allowed regions. Atomic coordinates have been deposited with the Protein Data Bank, accession code 1JF2.

2.4. Bioluminescence assay

The bioluminescence emission was measured with a home-made photometer or a Turner TD-20e luminometer by rapid injection of 10 μl of the photoprotein solution into the luminometer cell containing 1 ml of 100 mM $CaCl_2$, 100 mM Tris–HCl, pH 7.0, at room temperature.

2.5. Spectral measurements

Absorption spectra were obtained with a Hewlett-Packard 8453 UV-visible spectrophotometer. Bioluminescence and fluorescence spectra were measured with an SLM-8000 spectrofluorometer. Emission spectra were corrected by reference to the absolute spectral distribution of quinine sulfate, which was also used as the reference for the fluorescence quantum yield. The bioluminescence spectra were measured at 20°C from the obelin in 1 mM EDTA, 10 mM Tris–HCl, pH 7.0, and initiated by injection of $CaCl_2$. The concentration of free calcium was 0.5 μM in order to provide a constant light level during the spectral scan. The concentration of calcium was calculated with the MAXICHELATOR program.

3. Results

Fig. 1 shows the bioluminescence emission from W92F obelin mutant together with the fluorescence of the product. The left panel is the uncorrected bioluminescence spectrum to demonstrate that the band at shorter wavelength is much more enhanced than for the W86F aequorin published by Ohmiya et al. [8]. The right panel shows that after correction,

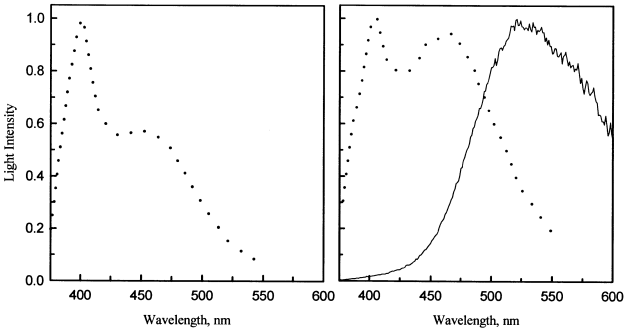

Fig. 1. Bioluminescence and fluorescence spectra. Left panel: Uncorrected bioluminescence spectrum (dotted line) from W92F obelin shows a much enhanced 400-nm contribution over that of aequorin W86F published by Ohmiya et al. [8]. Right panel: Corrected bioluminescence (dotted line) from W92F obelin and fluorescence (solid line) of the Ca^{2+}-discharged protein product ($\lambda_{max} = 510$ nm; Ex = 350 nm).

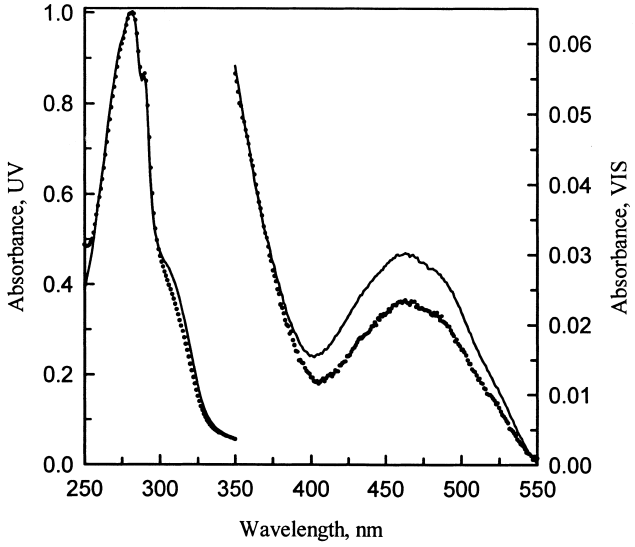

Fig. 2. Absorbance spectra of WT-obelin (dotted line) and W92F (solid line) both normalized at 280 nm.

the two bands are about equally intense, the bioluminescence maxima are at 410 and 470 nm. Probably the longer wavelength band is the same as for WT obelin (485 nm) only distorted by the tail of the shorter wavelength band.

The product fluorescence does not correspond to the bioluminescence emission (right panel) as also found for WT obelin ($\lambda_{max} = 510$ nm). For aequorin the fluorescence of the Ca^{2+}-discharged corresponds to the bioluminescence but Ohmiya et al. [8] did not report on the case with Ca^{2+}-discharged W86F-aequorin.

Fig. 2 shows that the absorption spectra of W92F mutant and WT obelin have the same maxima, 275 and 460 nm, with the visible absorbance relative to that at 275 nm being lowered in the mutant ($F_{1\,cm}^{0.1\%}$ at 280 nm is 1.99 and 2.5 for the W92F and WT obelin, respectively) since one Trp was replaced for Phe. The Ca^{2+}-discharged proteins also have the same maxima, at 344 and 278 nm in the presence of Ca^{2+}. The uncorrected excitation maximum of the Ca^{2+}-discharged proteins is around 340 nm. The fluorescence emission anisotropy is 0.33 and constant over the 340-nm excitation band indicating that the fluorophore is rigidly bound to the protein.

The overall structure of the W92F mutant of obelin shows the same sets of helix–turn–helix motifs as WT obelin, with an r.m.s.d. between the Cα-positions of the two proteins of only 0.525 Å. Details of the crystallization and structure solution will be given elsewhere. Of more relevance to the spectral properties is a comparison of the structures of the coelenterazine–oxygen binding site. The electron density map in Fig. 3 shows a relatively weak density around the coelenterazine C2-position, consistent with the substitution by a peroxy group the same as observed for aequorin by Head et al. [5], but in contrast to the single oxygen at this position for the WT obelin.

Fig. 4 is the substrate in the active site and compares the distances to the residues forming the binding cavity between the WT obelin and W92F. Apart from the insertion of a second oxygen between the C2-position and the Y190, the mutation produces no significant change among the other separations including the two nearby water molecules.

It also needs to be noted that the W92F mutant does not have significant differences in the specific activity, in the level of calcium-independent luminescence, in the temperature stability, and in the kinetics of coelenterazine binding (data not shown), in comparison with WT obelin.

4. Discussion

Proteins in this Ca^{2+}-regulated super-family, undergo a conformation change on binding Ca^{2+} and so it can be reasonably supposed that an analogous change is responsible for triggering the chemical reaction leading to the coelenteramide excited state from which the bioluminescence emission occurs. Indeed nuclear magnetic resonance evidence indicates that the obelin apo-protein can assume several conformations including that of obelin itself (with coelenterazine), of the Ca^{2+}-discharged protein (with coelenteramide) in the presence of Ca^{2+}, and of the same after removing Ca^{2+} [13]. The bioluminescence intensity reflects the overall reaction rate and this intensity reaches a maximum on the millisecond time scale, a time sufficient for an adjustment of the protein structure, particu-

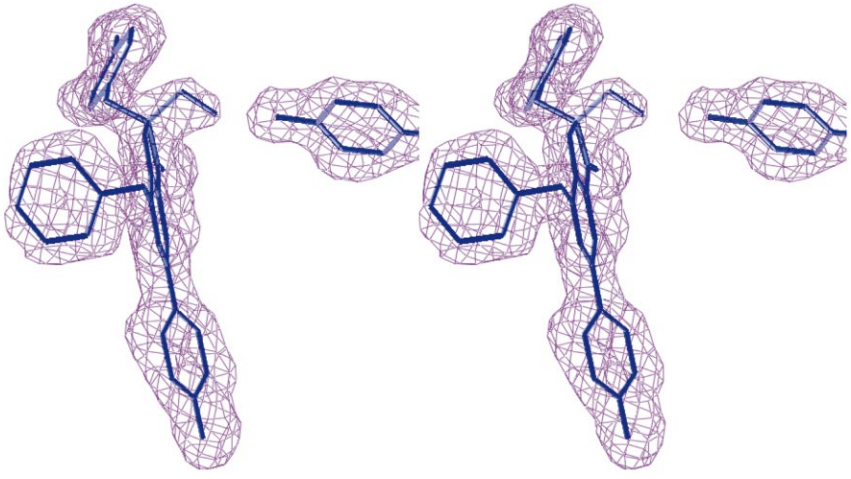

Fig. 3. Stereoview of the electron density map and substrate structure including residue Y190, within the binding cavity of W92F obelin. There is sufficient electron density around the C2-position of coelenterazine to account for a peroxy substitution. The electron density is weaker here than over the rest of the molecule as also observed in aequorin by Head et al. [5].

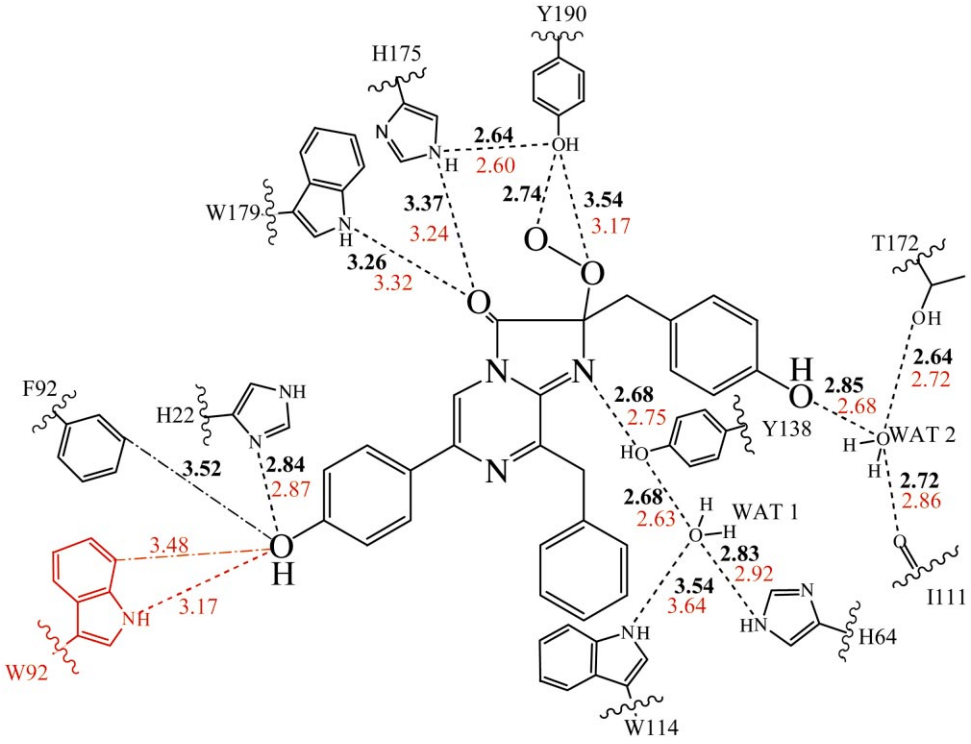

Fig. 4. Two-dimensional picture showing that the W92F mutation produces no significant change in the dimensionality of the 2-peroxycoelenterazine within the photoprotein binding site. Distances are in Å: red, WT-obelin; bold, W92F.

larly if only a minor alteration around the active site is involved.

The problem here is that if we seek an explanation for the difference in the bioluminescence spectra in structural terms, we only have the structures of aequorin, obelin and the W92F obelin mutant, in hand. In the overall structure of W92F obelin which will be presented in more detail elsewhere, there are some slight (0.5) displacements of some residues compared with WT obelin. In the binding cavity however, Fig. 4 shows that there is no significant (±0.3 Å) dimensional difference between WT obelin and the W92F mutant. The equivalent dimensions in aequorin are also nearly the same as these obelins. Therefore, how is the strong emission from **II** so favored in the W92F mutant? Besides the appearance of the C2-peroxide substitution in W92F mutant, the controlling factor must be in the presence or absence of the Trp92 hydrogen bond to the *p*-hydroxy group of the 6-phenyl substituent (Fig. 4).

Studies of the chemiluminescence in an aprotic medium as a model, indicate that **III** is the primary excited state produced by the bioluminescence reaction [14,15] (Scheme 1). In the ground state an amide hydrogen is not acidic but in the protein binding site the protonation rate of **III** might not be competitive with the rate of fluorescence transition. But in fact the bioluminescence spectrum from obelin but not aequorin, already shows a small contribution at 400 nm, identified as emission from **II**. The excited amide presumably receives its proton from the proximate hydroxy group of Y138. The W92F change removes the proton coupling via the hydrogen bond from the indole in the vicinity of the hydroxy of the 6-phenyl. Because the conjugation in the coelenteramide extends from this hydroxy to the N1-position, it would not be surprising to find an influence on the proton coupling between the N1 and Y138 hydroxyl. This proposition does not require any dimensional change although we have no knowledge of how the protein structure might have changed by the time the initially excited coelenteramide is formed. In the aequorin study of a number of Trp mutants, only the equivalent W86F mutant showed a clear bioluminescence spectral contribution from **II** although it is much smaller than in the case of W92F-obelin.

For aequorin the Ca^{2+}-discharged protein has a fluorescence spectrum corresponding to the bioluminescence. This would occur if there was a p*K* shift of the excited coelenteramide within the binding site to favor dissociation of the amide proton. However, for both Ca^{2+}-discharged WT obelin and W92F mutant the final state has a green fluorescence, maximum 510 nm. For obelin therefore, there must be a further structural change going to the final product state. Consequently, a direct spectroscopic examination of the amide anion in Ca^{2+}-discharged obelins is not experimentally accessible but could be feasible for the Ca^{2+}-discharged W86F aequorin.

Further structural and spectroscopic investigation of photoprotein mutants are planned to follow up these ideas.

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