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Spectral tuning of obelin bioluminescence by mutations of Trp92

Natalia P. Malikova[a], Galina A. Stepanyuk[a], Ludmila A. Frank[a], Svetlana V. Markova[a,b], Eugene S. Vysotski[a,b], John Lee[b,*]

[a] *Photobiology Lab, Institute of Biophysics, Russian Academy of Sciences Siberian Branch, Krasnoyarsk 660036, Russia*
[b] *Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA*

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Abstract **The Ca^{2+}-regulated photoprotein obelin was substituted at Trp92 by His, Lys, Glu, and Arg. All mutants fold into stable conformations and produce bimodal bioluminescence spectra with enhanced contribution from a violet emission. The W92R mutant has an almost monomodal bioluminescence (λ_{max} = 390 nm) and monomodal fluorescence (λ_{max} = 425 nm) of the product. Results are interpreted by an excited state proton transfer mechanism involving the substituent side group and His22 in the binding cavity.**

Key words: Photoprotein; Aequorin; Calcium; Fluorescence

1. Introduction

The calcium ion is one of the most important intracellular messengers. The Ca^{2+}-regulated photoproteins, of which aequorin [1] is the best-known example, have been used for intracellular calcium studies for more than 30 years. We have recently shown that obelin derived from the marine hydroid *Obelia* [2,3] has certain properties that could make it even more attractive as an intracellular calcium probe.

Aequorin and obelin are classified as 'photoproteins' because they require only addition of Ca^{2+} to trigger the bioluminescence, distinguishing them from the luciferases that have the normal enzyme requirement for the addition of substrate [4]. Photoproteins generate a blue bioluminescence with a broad spectral distribution and species-dependent maximum, 465 nm for aequorin [1], and the range 475–495 nm for various obelins [3,5]. The light reaction is an oxidation of a tightly but non-covalently bound ligand called coelenterazine (Fig. 1, top structure) to form the excited state product called coelenteramide [6].

Aequorin Trp mutants were made by Ohmiya et al. [7] and one of them with substitution of Trp86 to Phe produced a bimodal bioluminescence with the second band maximum at 410 nm. Fluorescence studies of coelenteramide and its analogs in solution have established this 410-nm band as originating from the excited state of the neutral form [8] (Fig. 1). Residue Trp92 in obelin is the one corresponding to the position mutated in aequorin to give bimodal emission and it was found that W92F obelin also yielded a bimodal bioluminescence, except with a more prominent shorter wavelength band at 405 nm, so that the bioluminescence color was visibly violet [9]. In an attempt to understand why the substitution of this particular amino acid residue favors the formation of the neutral species of the excited state of coelenteramide, the spatial structure of the W92F mutant was determined [9,10]. The crystal structures showed no significant difference in the dimensions of the active sites of wild-type (WT) [11] and W92F obelins [9,10] so it was proposed that the bioluminescence spectral shift arises from removal of a hydrogen bond from the indole of Trp92 to the 6-*p*-hydroxyl group (Fig. 1). This hydrogen bond would stabilize the ion-pair excited state favoring longer wavelength emission and that is why its removal produces violet bioluminescence and a bimodal emission spectrum. Hence a proton-relay mechanism has been proposed for Ca^{2+} triggering of bioluminescence chemistry and for excited state proton transfers that explain the production of different bioluminescent spectra [10].

In this paper we report studies of obelin mutants with substitution of Trp92 to His, Arg, Lys, and Glu which side chains possess different donor–acceptor hydrogen bond and charge properties. In each case bioluminescence and fluorescence spectral properties are affected and qualitative rationalizations are presented based on the hypothetical mechanism (Fig. 1). A practical outcome derived from these studies is a fundamental knowledge enabling us to tune the bioluminescence spectral distributions to provide construction of photoprotein probes with distinct spectral signals that could provide synchronous measurements of calcium transients in different cell compartments. Of particular note is that one of these obelin mutants, W92R, displays a monomodal violet bioluminescence with λ_{max} = 390 nm.

2. Materials and methods

2.1. Molecular biology

Site-directed mutagenesis was done on the template pET19-OL8 *Escherichia coli* expression plasmid carrying the *Obelia longissima* WT apo-obelin [13]. Mutations resulting in the amino acid change – W92R, W92K, W92H, or W92E – were carried out using the QuickChange site-directed mutagenesis kit (Stratagene, USA) according to the protocol supplied with the kit. The plasmids harboring mutations were verified by DNA sequencing. Site-directed mutagenesis is a more practical method for photoproteins than random mutagenesis as has been used for producing color mutants of the green fluorescent protein. The reason is that the apophotoprotein has to be charged with coelenterazine under calcium-free conditions, very difficult to achieve with *E. coli* colonies on a Petri dish.

For protein production, the transformed *E. coli* BL21-gold was cultivated with vigorous shaking at 37°C in Luria–Bertani (LB) me-

*Corresponding author. Fax: (1)-706-542 1738.
E-mail address: jlee@bmb.uga.edu (J. Lee).

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dium containing ampicillin and induced with 1 mM isopropyl thiogalactose (IPTG) when the culture reached an OD_{600} of 0.5–0.6. After addition of IPTG, the cultivation was continued for 3 h.

2.2. Purification of mutant obelins

The obelin mutants were purified and charged with coelenterazine as previously reported for WT recombinant obelins [2,3]. The final products were homogeneous according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). We observed that the efficacy of charging with coelenterazine varied for the different mutants with W92R showing the lowest yield for example. However, in the last step of purification by ion-exchange chromatography the photoproteins were clearly separated from the uncharged protein [3]. All mutants used were fully charged as confirmed by their absorption spectra.

2.3. Ca^{2+}-discharged mutant and WT obelins

To prepare samples the concentrated solutions of photoproteins were diluted 10 times with 50 mM Bis-Tris propane pH 7.0 containing $CaCl_2$ (final concentration of calcium in a sample = 1 mM). Fluorescence was measured after bioluminescence reaction ceased.

2.4. Bioluminescence assay

The bioluminescence intensity was measured with a BLM 8801 photometer (SCTB 'Nauka', Russia) by rapid injection of 0.2 ml of 100 mM $CaCl_2$, 100 mM Tris–HCl pH 8.8 into the photometer cell containing 0.5 ml of 5 mM ethylenediamine tetraacetic acid (EDTA), 100 mM Tris–HCl pH 8.8 and the photoprotein aliquot.

2.5. Spectral measurements

Absorption spectra were obtained with an UVIKON 943 Double Beam ultraviolet-visible (UV/VIS) spectrophotometer (Kontron Instruments, Italy). Bioluminescence and fluorescence spectra were measured with an AMINCO spectrofluorimeter (Thermo Spectronic, USA). Emission spectra were corrected with the computer program supplied with the instrument. The bioluminescence spectra were measured in 1 mM EDTA, 50 mM Bis-Tris propane buffer pH 7.0 and initiated by injection of $CaCl_2$ solution in the same buffer. The con-

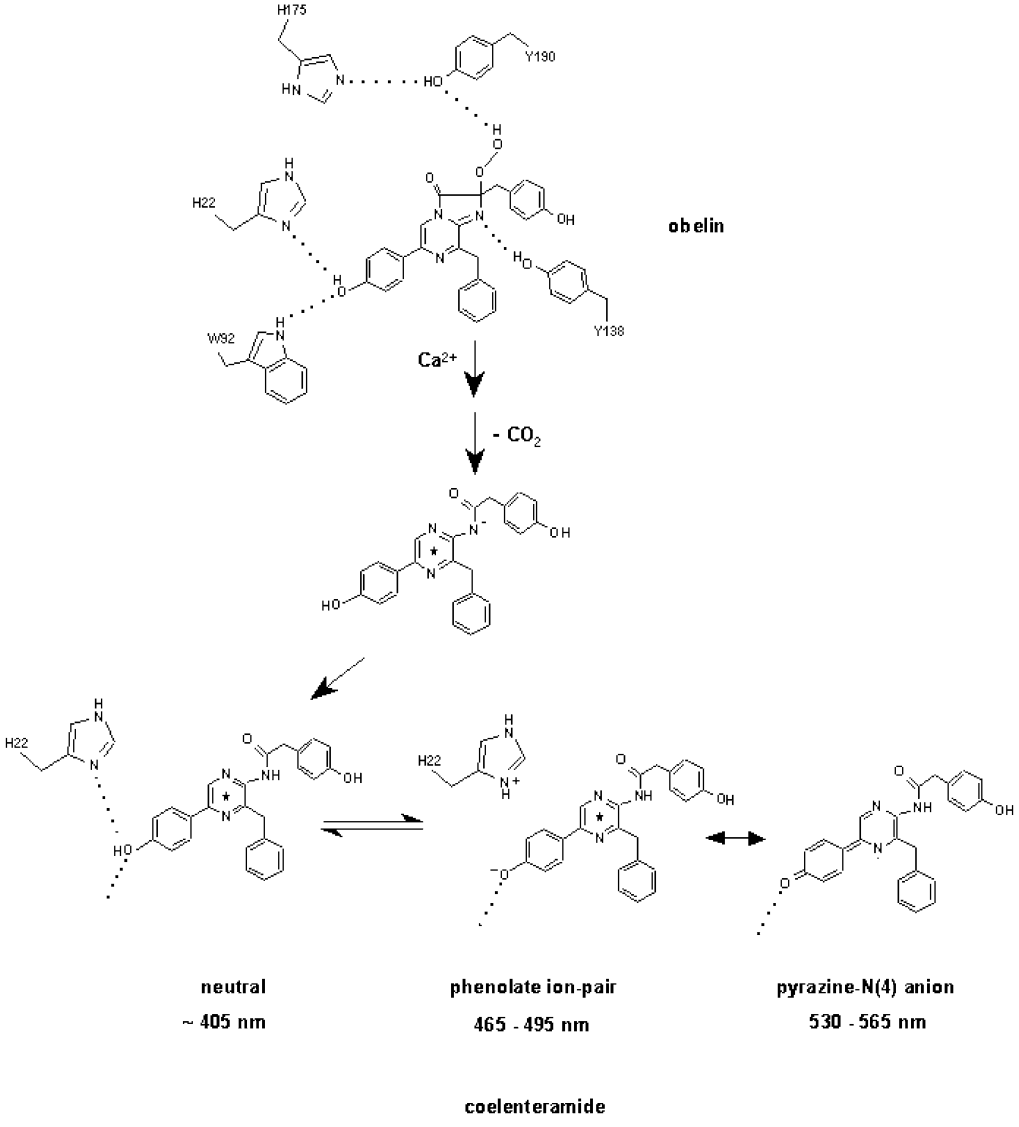

Fig. 1. Coelenterazine 2-hydroperoxide has hydrogen bond (...) interactions with several residues in the binding cavity of obelin. Addition of Ca^{2+} triggers the oxidative decomposition according to the model studies of McCapra and Chang [12], to form the coelenteramide amide anion as the primary excited state (*). Subsequent excited state proton transfer is proposed to transform this into the excited phenolate ion-pair [10] responsible for bioluminescence emission at longer wavelength, λ_{max} = 465–495 nm. The neutral coelenteramide yields the bioluminescence with λ_{max} = 405 nm for W92F obelin. The Ca^{2+}-discharged obelins show fluorescence in the green (λ_{max} = 510–520 nm) attributed to dominance of the pyrazine-*N*(4) anion resonance form as model studies show [8].

Table 1
Bioluminescence of obelins and fluorescence of Ca^{2+}-discharged proteins

Mutants	Bioluminescence		Fluorescence[a] (λ_{max} (nm))
	Violet/blue ratio[b]	Activity[c]	
WT	0.17	1.0	507
W92H	0.36	1.0	505
W92F	1.0	1.0	510
W92K	0.74	0.5	502
W92E	1.3	0.15	425
W92R	4.2	0.06	425

All at pH 7.0.
[a]Excitation at 350 nm.
[b]Violet/blue is the ratio of 390/485 nm spectral intensities.
[c]Relative to WT.

centration of free calcium was around 0.5 μM in order to provide an approximately constant light level during the spectral scans. The calcium concentration was estimated with the MAXICHELATOR program. All bioluminescence and fluorescence measurements were carried out at room temperature.

3. Results

The bioluminescence spectral distributions from all the obelins are bimodal and appear to be composed of two contributions, blue and violet. Even in WT obelin the violet contribution appears as a small shoulder [3,10]. In Table 1 the violet/blue ratio is a qualitative analysis of the effect of each residue substitution and it is clear that all substitutions of Trp92 favor the violet band to varying extents.

There is no apparent correlation between the properties of the substituted side group and the effect on the bioluminescence spectral distribution. The W92R bioluminescence is almost completely violet, ratio 4.2 and maximum 390 nm, the reason why the 390-nm intensity is used for the ratio measurement (Fig. 2). A clear result however is that the appearance of a violet band is accompanied by a sharp drop in bioluminescence activity and a shift of fluorescence from green to violet (Fig. 3). It needs to be noted that only the Ca^{2+}-discharged proteins have visible fluorescence and that fluorescence is

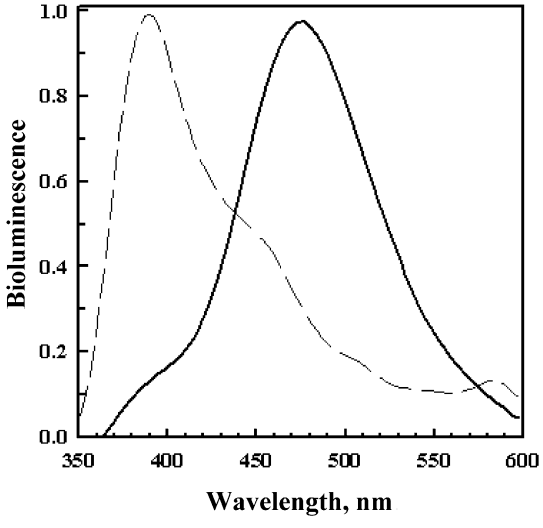

Fig. 2. Normalized bioluminescence spectra of WT (solid line) and W92R (dashed line) obelins at pH 7.0.

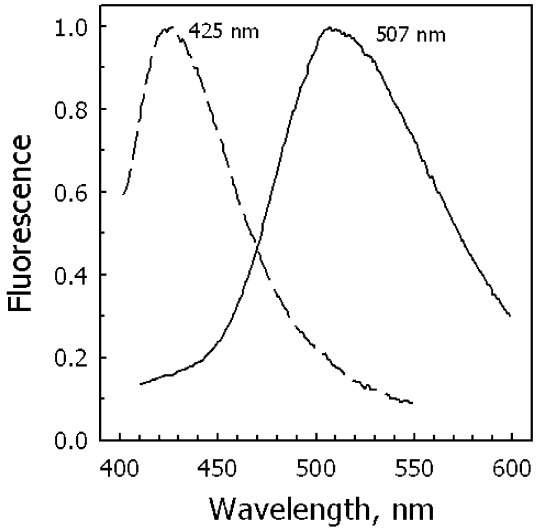

Fig. 3. Fluorescence of Ca^{2+}-discharged WT (solid) and W92R (dotted) obelins (pH 7.0; excitation at 350 nm).

monomodal not like bioluminescence. Nevertheless all these mutants fold into a stable conformation competent for bioluminescence activity also with a level of 'calcium-independent luminescence' approximately the same as that for the WT [2].

The absorption spectra of obelin mutants are all the same as the WT [3] with a maximum around 460 nm (not shown). Fig. 4 however shows that there are significant differences in the Ca^{2+}-discharged proteins with W92F and W92H producing a shoulder around 310 nm and a decrease in 350-nm absorbance over WT, and the near-UV absorbance almost absent in W92R and W92E. This loss of absorbance could be due to dissociation of coelenteramide and there is also a corresponding decrease in the 425-nm fluorescence intensity over that from the proteins with green fluorescence.

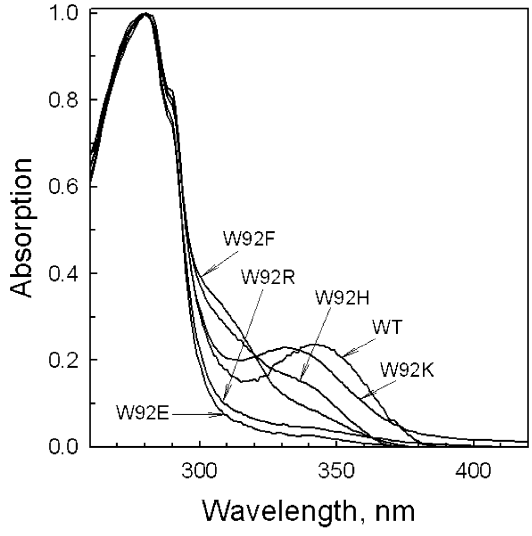

Fig. 4. The normalized absorption spectra of Ca^{2+}-discharged WT obelin and mutants at pH 7.0.

4. Discussion

From a detailed study of the spatial structure of obelin and its W92F mutant, we have proposed a proton-relay mechanism for the Ca^{2+} triggering of the bioluminescence reaction and for formation of different product excited states [10]. This hypothesis is elaborated in Fig. 1. The proposal is that Ca^{2+} binding results in change in the separation between His175 and Tyr190 causing dissociation of the hydroperoxide and in several steps leading to the excited state of coelenteramide anion. The chemical steps are the ones first demonstrated by McCapra and Chang [12] for the model chemiluminescence in aprotic solvent. The chemiluminescence has $\lambda_{max} = 455$ nm and the same spectral distribution as the fluorescence of the authentic compound, evidence that this anion is the emitter of chemiluminescence. However as amide anion has a very high p*K*, in the binding site of a protein, several nearby sources of protons should allow rapid proton transfer to produce the excited state of neutral coelenteramide.

The formation of a blue bioluminescence state is proposed to be due to excited state proton transfer from the *p*-hydroxyphenyl substituent of coelenteramide to His22 to form the phenolate ion-pair state. Recent model fluorescence studies conclude that the long wavelength bands, $\lambda_{max} = 465$ for aequorin, and $\lambda_{max} = 475$–495 nm for obelins, originate from excited phenolate states, although in this case fluorescence evidence is not so diagnostic, since environmental perturbations can be quite large. This proton transfer is feasible because the p*K** of phenols is known to be decreased several units below the p*K*, so that the p*K** of the hydroxyphenol at or below 6.5 would allow rapid proton transfer to His22. The H bond donated from Trp92 stabilizes the phenolate state with the result that the WT aequorin and the WT obelins show little or no bioluminescence from the neutral coelenteramide.

Without knowledge of spatial structures of these mutants we may advance only reasonable ideas to explain the observed effects. We assume that the dimensions of the binding cavity do not change for each substitution and using computer modeling we allow only the substituted side chain to vary its orientation. In each case out of a number of possible conformations there are two which seem more probable in that hydrogen bonding interactions are maximized. These are shown in Fig. 5 and fall into two groups, group A where the side chain orientation is to the hydroxyl group of coelenterazine, and group B, where the interaction is with the His22.

In conformation A for histidine in position 92 (W92H), the ND atom of His donates an H bond to the hydroxyl group of coelenteramide. As His should be a better donor than the Trp92 in the WT obelin, the expectation is that the bioluminescence should be the same as for the WT. On the contrary, the neutral coelenteramide band is enhanced in W92H (Table 1). In conformation B the His22 is fully satisfied by H bonding to His92 so another proton transfer from the excited neutral coelenteramide should not be likely. Thus the emission in the violet is enhanced by this orientation. The side chain of

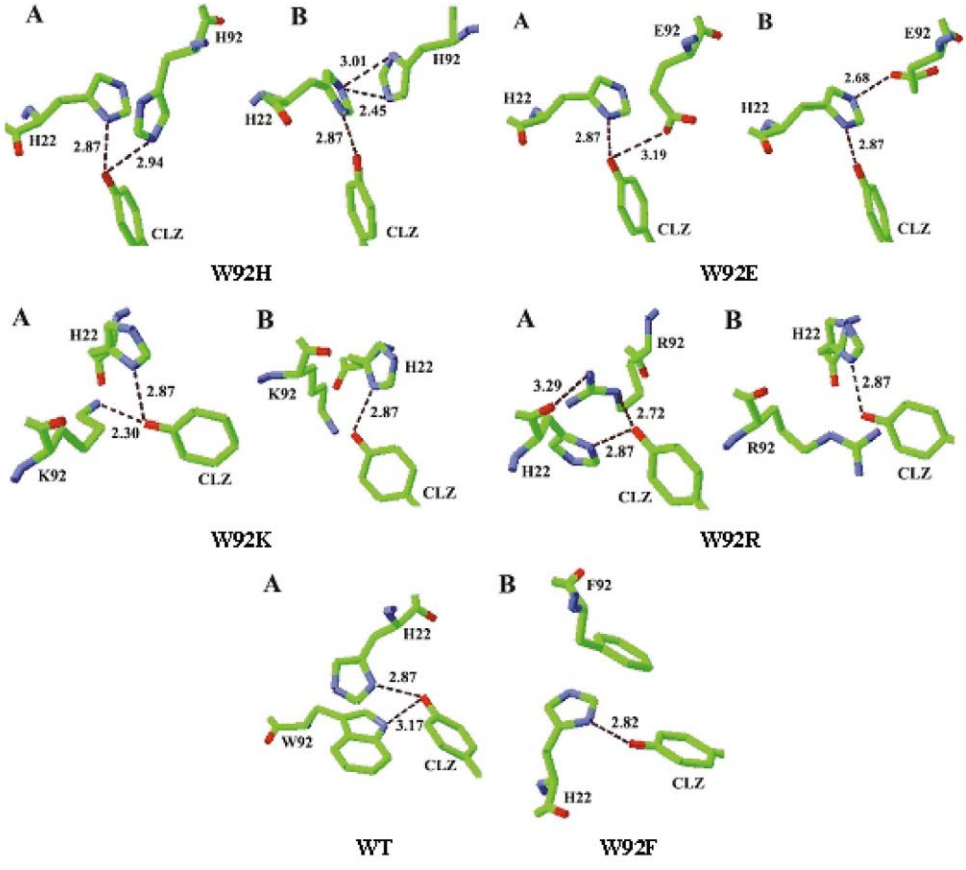

Fig. 5. The most probable two conformations of residues in mutated obelins and the structures of WT (PDB 1EL4) and W92F (PDB 1JF2) obelins. CLZ, 6-(*p*-hydroxy)-phenyl group of coelenterazine.

Glu in position 92 (W92E) which possesses negative charge at physiological pH, would be a good acceptor of the hydrogen from the hydroxyl of coelenteramide thus impeding the excited state proton transfer to His22 and the bimodal bioluminescence is thereby favored. For Lys and Arg, W92K and W92R, both are positively charged and in group A place their charge nearby the hydroxy group. However if they orient in conformation B, the positive charge now near the His22 would prevent the excited state proton transfer and the bioluminescence now is dominated by emission from the neutral excited coelenteramide.

The fluorescence spectra of each of the mutants are all single bands, either green or violet. The argument just given is also applicable for interpretation of violet fluorescence from the products of W92R and W92E. In these mutants there is also a concomitant loss of bioluminescence activity and this can be explained by a high exposure to the aqueous environment, as coelenteramide fluorescence is strongly quenched in water. The absorption spectra (Fig. 4) indicate that in these mutants the coelenteramide dissociates from the Ca^{2+}-discharged protein.

The identity of the coelenteramide excited state that gives the green fluorescence is not established but a recent proposal is that it is the pyrazine-*N*(4) anion resonance form of the coelenteramide anion [8]. It should be noted that for aequorin the bioluminescence and product fluorescence spectra overlap, but for obelin the fluorescence spectra have 25 nm longer wavelength. The coelenteramide also remains tightly bound (Fig. 4) and except for W92K, there is no loss of bioluminescence activity in those mutants with product green fluorescence. Therefore, in these cases we propose that His22 in the final product conformation remains in a position able to accept the proton from the excited fluorescent state of coelenteramide. The shift to longer wavelength could be the result of a more polar bound state environment in the final conformation.

In conclusion we show here four new Trp92 mutations of obelin from *O. longissima*, which are active and stable, and have changed bioluminescence and fluorescence properties. Bioluminescence maxima in either the violet or blue are found, or mixtures of these two bands. The fluorescence of the Ca^{2+}-discharged obelin mutants are monomodal bands either blue-violet, $\lambda_{max} = 425$ nm, or green, $\lambda_{max} = 502–510$ nm. The properties of these binding site mutants are consistent with proton transfer between the *p*-hydroxy-phenyl group and His22 as proposed [10]. Although we put forward simplistic explanations for the observed spectral properties of the mutants, it is only the three-dimensional structure of the binding site of each that can provide reality.

A practical outcome from these studies is that, on the basis of our proposed proton transfer mechanism for formation of the excited states, the knowledge of the spatial structure of the binding cavity, and the use of site-directed mutagenesis, we could construct photoprotein color probes that may be useful for multi-wavelength detection of intracellular calcium transients. Even now, the W92R mutant may be used for some types of double wavelength applications paired with WT obelin because the overlap of the bioluminescence spectra of these photoproteins is only around 20%.

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