Accurate Color Tuning of Firefly Chromophore by Modulation of Local Polarization Electrostatic Fields

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Although many microenvironmental factors contribute to the color shift of light emission from the firefly chromophore, the dominant one is the local electrostatic field. This opens up the possibility of accurate color tuning the bioluminescent absorption and emission by adjusting the local charged residues. With this aim, the optical response of oxyluciferin for different electrostatic fields is computed by using time-dependent density-functional theory. We find that the wavelength shift is correlated to the projection of the electrostatic field on the molecular plane, and that the fluorescent intensity of the second excitation peak can be effectively enhanced or suppressed (±30%) by field modulation. A model is formulated by correlating the shift in the spectral maxima with the projection of the local electrostatic field on the molecular plane. This method provides a predictable determination of the structural modifications leading to a particular color shift and/or fluorescent efficiency enhancement.

One of the best known bioluminescent systems, the firefly fluorescent protein, has been used in the past years as a unique marking tool, widely applied in bioimaging,[1] as a reporter for gene expression,[2] in HIV dynamics,[3] in the motion of single-molecule motor,[4] and in biosensors for environmental pollutants.[5] However, in order to perform real-time monitoring of several different events at the same time (both in vitro and in vivo), for special targeting cases, or in complex biochemical systems, one often requires the reporter to be designed or modified for emitting light at specific wavelengths. For this purpose, bioengineering techniques such as mutagenesis have been employed to produce color-shifted mutants of the firefly luciferase.[6,7] A number of color shifts have been observed as a result of the structural modification of the protein. However, due to the ambiguity and complexity of the microenvironmental effects on the emitted light of the chromophore, really intentional and accurate wavelength modulation has yet to be achieved.

The chromophore of the firefly fluorescent system, oxyluciferin, is located in the central part of a protein pocket and is surrounded by several side chains.[8−10] There are two possible ways that environmental effects can alter the absorption and emission properties of the chromophore, either (i) by modifying the geometry of the chromophore or (ii) through the electrostatic potential created by the nearby residues. It was proposed that for the firefly system the dominant effect was (ii), i.e., that charged residues and polar solvents would play the key role in controlling the wavelength and luminescence intensity.[11−13] To study this effect, we mapped each residue to an equivalent electric field in order to describe its microenvironmental interactions with oxyluciferin (see Figure 1). Taking this equivalent field as an intermediate quantity, we can try to

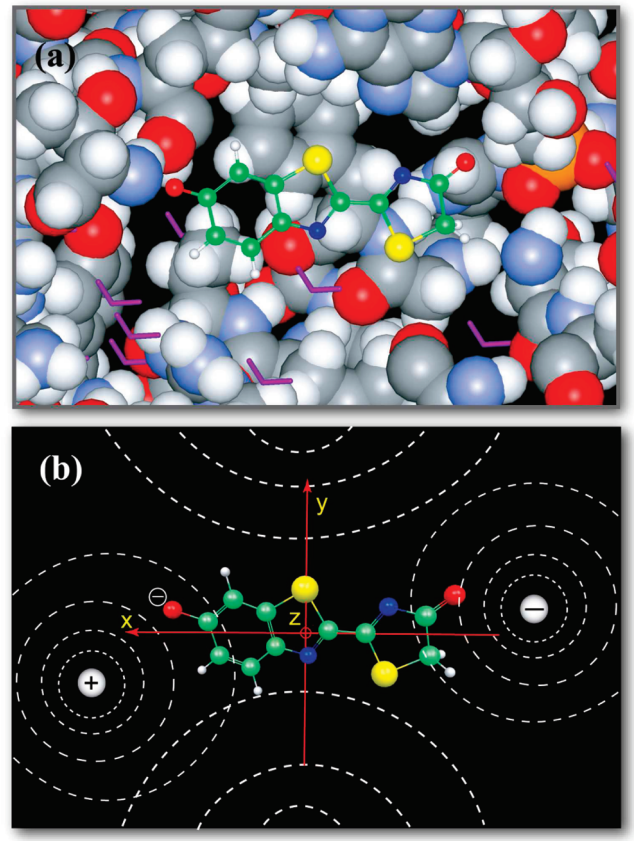

Figure 1. (a) Keto(−1) oxyluciferin with the surrounding microenvironment and (b) schematic of the Coulomb interaction, which can be represented by the effective electrostatic fields. Three perpendicular axes, *x*, *y*, and *z*, are defined.

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TABLE 1: Contribution Ratio *p* and Oscillator Strength *f* of HOMO → LUMO Transitions for Main Spectral Peaks, A1 and A2, under Different Electrostatic Fields *E*

	p (*f*)		
E (mV/Å)	HOMO → LUMO (A1)	HOMO − 2 → LUMO [A2(1)]	HOMO − 4 → LUMO [A2(2)]
		E_x	
−3	84% (0.356)	51% (0.035)	40% (0.351)
0	81% (0.336)	54% (0.294)	63% (0.139)
3	78% (0.309)	72% (0.400)	85% (0.081)
		E_y	
−3	82% (0.366)	47% (0.247)	55% (0.147)
0	81% (0.336)	54% (0.294)	63% (0.139)
3	81% (0.308)	60% (0.344)	65% (0.116)

establish a quantitative connection between the environmental effects and the fluorescent color shifts. On the basis of this connection, one can then achieve accurate color tuning by establishing appropriate mutation strategies.

Broad studies of the chromophores of firefly fluorescent protein in both bioluminescence and chemiluminescence ascertained that the keto-form oxyluciferin is the most important light emitter responsible for the fluorescence.[14−16] Therefore, we focused our investigations on the deprotonated keto(−1) form of the oxyluciferin molecule (Figure 1a), on the basis of the X-ray structure of the Japanese firefly luciferase (*Luciola cruciata*, PDB database code: 2d1r).[9] We then performed systematic *ab initio* calculations within time-dependent density functional theory (TDDFT) of the chromophore, modeling environmental effects through a static electric field, as explained above. The polarization electrostatic field was applied to the oxyluciferin molecule in three perpendicular directions: *x* along the long molecular axis, *y* along the short axis, and *z* normal to the molecular plane, as indicated in Figure 1b. TDDFT calculations, the already proven approach for the study of biochromophores,[17] were carried out using octopus code.[18] Exchange-correlation effects were treated, adiabatically, within the local-density approximation (LDA).[19,20] Hybrid (B3LYP[21,22]) calculations were also performed to assess the accuracy of the LDA spectra (see the Supporting Information). The changes in the spectra were not significant (<0.02 eV), thus validating the much less computationally demanding LDA approach. It is important to note that the absorption spectrum (especially the first excitation) often reflects the main features of the optical properties of the inverse action, light emission, though a red shift will take place after the excited state relaxation. In fact, the emission probability can be simply described by the van Roosbroeck−Shockley relation,[23] as a function of the absorption rate α: $R(\nu) = \rho\alpha(\nu)$, where ρ is the photon density that is given by $8\pi\nu^2 n^3/c^3$.

There are two main excitation peaks of the gas-phase keto(−1) form: The first one (A1) appears in the green region (2.43 eV) whereas the second one (A2) is in the violet region (3.25 eV). These two peaks have similar intensities, indicating a possible bicolor emitter. This means that detection of two targets using the same reporter could be achieved by setting separate band-pass filters for different emitted light colors. Detailed inspection of the contribution of transition components (Table 1) shows that the A1 peak can be assigned dominantly to the HOMO → LUMO transition, whereas the A2 peak can be assigned to the HOMO − 2 → LUMO transition. In addition, a small shoulder of A2 is present on the higher energy side, originating from the deeper HOMO − 4 → LUMO transition.

In order to evaluate the magnitude of the real electrostatic fields on the oxyluciferin site induced by the microenvironment, we performed a series of zero-field ground-state calculations taking into account some representative amino-acid residues (mainly the charged ones) close to the oxyluciferin molecule. Table 2 shows the closest distance between each residue and the oxyluciferin, and the electrostatic field in the molecular plane for the indicated charge state. On the basis of the range of magnitudes found, we decided to scan the shifts in the absorption peaks with electric fields ranging from −150 to 150 mV/Å. The shift of spectral peaks A1 and A2 as a function of the electric field is shown in Figure 2. The electric field is shown to play a key role in the color shift of the emitted light, especially for fields in the molecular plane (*x* and *y* direction). On the contrary, the influence, either on the wavelength or the intensity of the spectra, by the electrostatic field parallel to the *z* axis is negligible. This implies that those environmental factors without an effective component on the oxyluciferin molecular plane, for example out-of-plane residues or solvent molecules, can be excluded when looking for the main environmental contributions to the optical properties. In contrast, charges on the molecular plane are of extreme importance in the modification of the emission properties. This leads to a considerable simplification of the microenvironment analysis.

TABLE 2: Average Electrostatic Field Imposed by Selected Important Residues Close to the Oxyluciferin[a]

residue	number	charge	*d* (Å)	*e* (mV/Å)
Lys	531	+1	5.15	59.1
Arg	339	+1	6.17	−52.9
His	247	+1	4.54	103
His	247	0	4.54	8.5
H_2O	2325	0	3.33	−6.7
H_3O	2325	+1	3.33	−109

[a] The *d* stands for the shortest distance between the residue and the oxyluciferin, and *e* is the average magnitude of electric field in *x* axis.

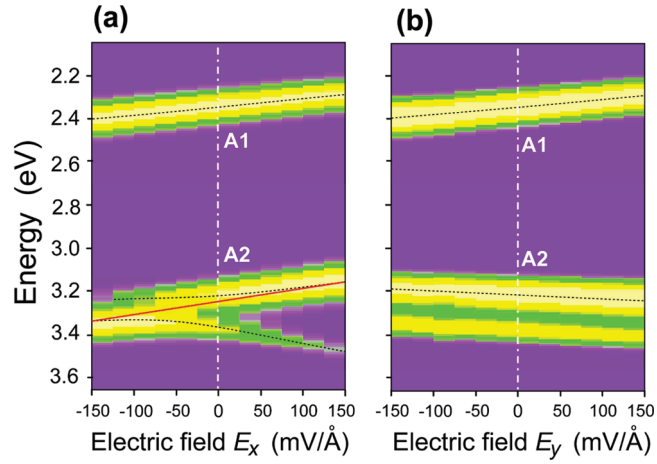

Figure 2. Contour plots of the absorption peaks A1 and A2 showing the color shifts as a function of the external electrostatic fields along (a) *x* and (b) *y*.

One can see in Figure 2 that the A1 peak shows a distinct linear wavelength shift as a function of the electric field applied in the in-plane axes. For E_x in Figure 2a, the increase of the electrostatic field gives rise to a red shift of the A1 peak. A 300 mV/Å field leads to a 0.15 eV peak shift. Similarly, increase of E_y by 300 mV/Å results in a red shift of 0.11 eV. These results strongly support the claim that the Coulomb interaction is the main factor contributing to the experimental observation of a color shift by mutagenesis insertion of charged amino acids.[11,12] With our calculations, this claim is now quantitatively

supported. On the other hand, the linear and continuous dependence of the wavelength variation on the electrostatic field indicates the feasibility of establishing new biomolecular techniques for intentional color modulation through control of the local polarization electric field.

This simple model can be readily used to explain and predict the wavelength shift in protein designs by the site-directed mutagenesis method. For example, for the variant protein of the North American firefly, a mutation (Q283R) where a glutamine is replaced by an arginine leads to a shift from 555 to 560 nm at pH 8.0.[24] As this residue is at 12.6 Å from the oxyluciferin, the electrostatic field induced in the molecular plane can be estimated to be 28.5 mV/Å, which corresponds to a 4 nm red shift according to the results in Figure 2, in excellent agreement with the experimental result. Another similar examination was done to the R218Q (4.46 Å from the chromophore plane) mutant at pH 8.0, which leads to an emission spectral shift from 557 to 608 nm.[25] We estimate an in-plane electrostatic field of 298 mV/Å, and by extrapolating the linear fit in Figure 2 to higher fields, we get a red shift of 49 nm, again in good agreement with the experimental value.

The role and influence of a solvent effect (e.g., water molecules) has been widely discussed, but it still remains ambiguous. There is, in fact, a water molecule close to the oxyluciferin (see Table 2). However, if one considers the water to be neutral, the electrostatic field it induces in the chromophore is negligible, and so it can not play a key role in altering the emission color of the fluorescent system. Of course, if the water molecule is protonated, its polarity and electrostatic contribution will be considerably larger leading to a noticeable shift in the spectra.

The second excitation peak, A2, has two components. The two subpeaks split for positive E_x. During the splitting, the strength of the main subpeak remains the same while the shoulder on the higher energy side gradually fades out. Although the components split, the overall trend of the A2 wavelength shift is distinctly governed by the main spectral maximum. Apparently, this maximum still depends linearly on the electric field, as indicated by the red line in Figure 2a, the slope of which is close to that of the A1 shift. On the basis of these results, we can conclude that the electrostatic field along the long molecular axis will lead to a systematic red shift of oxyluciferin luminescence. In contrast, the trends of the A1 and A2 wavelength shifts with E_y are opposite to each other, as shown in Figure 2b. Peak A1 undergoes a red shift with increasing E_y, whereas peak A2 experiences a blue shift. The opposite trends of the shifts can be useful for color tuning in special cases. Careful selection of E_x and E_y can allow for the modulation of the A1 color only, with the A2 color remaining constant through mutual cancellation of the shifts induced by E_x and E_y.

Further explanation of the origin of the shifts requires looking at the properties of the molecular states involved. The electrostatic-field influence in the main molecular states involved in the optical transitions A1 and A2 is shown in Figure 3. One can see that the field along the long molecular axis has a larger impact than the field along the short molecular axis. This is not surprising, since the long molecular axis is in the direction of strong polarizability, which naturally leads to a more sensitive response to the external electric field. On the other hand, the energies of LUMO states are less sensitive than HOMO states, which suggests that the spectral wavelength shifts observed above should be associated dominantly with the energetic shift of the occupied molecular states. A detailed inspection shows

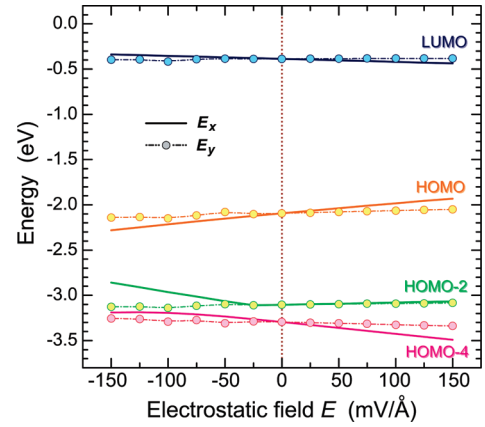

Figure 3. Variation of the main molecular states involved in optical transitions of A1 and A2 as a function of electrostatic fields. Solid lines are variations under E_x and dash−dot lines under E_y.

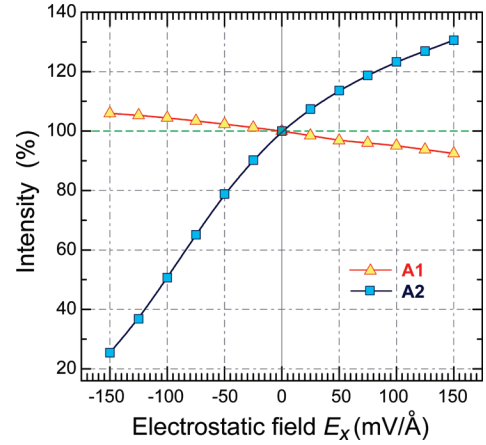

Figure 4. Spectral intensities of A1 (△) and A2 (□) peaks as a function of E_x. The peak intensities are normalized by the intensity at zero field.

that the HOMO energy increases with increasing electrostatic field, which consequently results in the distinct red shift of A1. As discussed previously, the HOMO − 2 and HOMO − 4 states are responsible for the transitions of the A2 peak. It was found that the decrease of the HOMO − 4 energy for positive E_x plays the key role in the subpeak splitting (Figure 2a). Although HOMO − 2 increases significantly for negative E_x, the fast drop of the transition probability (oscillator strength) of HOMO − 2 → LUMO makes the subpeak decay quickly, and consequently, the possible splitting in this negative field region disappears. Another interesting feature is that the energy gap between HOMO − 2 and HOMO − 4 closes slightly for negative E_y, which, in fact, effectively narrows the line width of the A2 peak, indicating a way of improving the monochromaticity of emitted light.

Another important issue in fluorescence is the light emission efficiency. Figure 4 shows the spectral intensities as a function of E_x, normalized by the intensity at zero field. A1 shows a linear variation within ±10%, whereas A2 changes much more drastically upon electric field modulation. The efficiency of A2 is strongly suppressed by the negative field while effectively enhanced by the positive field. In detail, with an electrostatic field of 150 mV/Å, the intensity can be improved by nearly 30%, which indicates an efficient enhancement mechanism of the fluorescent brightness of firefly chromophore. This again corroborates the proposed mechanism for modification of bioluminescence intensity in mutagenesis experiments.[13] Moreover, the difference in the electric-field sensitivity of A1 and

A2 again suggests that careful local electrostatic field design allows for switching on and/or modulation of the modes of monocolor and bicolor absorption or emission of fluorescent molecules for special marking needs.

In conclusion, we performed TDDFT calculations of the absorption spectra of the firefly light emitter, oxyluciferin, under various local electric fields. The wavelength shifts of the first two excitation peaks (A1 and A2) show a simple linear relationship with the electrostatic field. This linear relationship can be easily used to explain and predict the shift in the absorption maximum of mutants of the firefly luciferase. In addition, we found that the A2 intensity varies drastically in the presence of the *x*-axis electric field, indicating an existing room for light efficiency improvement. Thus, a complete guide map for the accurate color tuning and efficiency enhancement was established. On the basis of this map, special reporter colors can be obtained by careful design of chromophore microenvironments reproducing the required electrostatic fields. Work along this line is in progress.

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Supporting Information Available: Technical details, spectra with different functionals, and emission spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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