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Color Hues in Red Fluorescent Proteins Are Due to Internal Quadratic Stark Effect

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Abstract

Intrinsically fluorescent proteins (FPs) exhibit broad variations of absorption and emission colors and are available for different imaging applications. The physical cause of the absorption wavelength change from 540 nm to 590 nm in the Fruits series of red FPs has been puzzling because the mutations that cause the shifts do not disturb the π -conjugation pathway of the chromophore. Here we use two-photon absorption measurements to show that the different colors can be explained by quadratic Stark effect due to variations of the strong electric field within the beta barrel. This model brings simplicity to a bewildering diversity of fluorescent protein properties, and it suggests a new way to sense electrical fields in biological systems.

The initial cloning and characterization of the Green Fluorescent Protein (GFP) (1,2) soon led to the mutants that produce blue, cyan and yellow fluorescent proteins (FPs) (3). These are powerful genetically-encoded tools for imaging living cells and tissues. The first protein fluorescing in the red part of the spectrum (DsRed) was discovered more recently (4), and many different hues of red fluorescent proteins (RFPs) now exist (5). The most widely used RFPs are the Fruit series (6,7), and they are particularly well suited for deep tissue and multicolor imaging. Although it is clear that different chromophore structure (e. g. in GFP versus DsRed) will result in different absorption wavelengths (8), the large spectral shifts (up to ~50 nm) observed in the Fruit series are due to mutations in the staves of the beta barrel that surround the RFP chromophore and do not disturb its conjugation pathway (see crystallography data (9–12)). How can only a few such perturbations produce dramatic changes in the chromophore optical properties?

Shu et al. (10) suggested that the red shifts of absorption peaks in mStrawberry and mCherry, compared to DsRed, are due to amino acid substitutions that result in rearrangements of hydrogen bonds, and redistribution of charges, close to the chromophore. This can cause the perturbation of local electric field inside the protein. In other FP series, where the mutations do not disturb the chromophore conjugation pattern, spectral shifts are also thought to arise from changes in local electrostatic interactions between the chromophore and surrounding (13–15). Despite these indications that electrostatics can play an important role in color

Supporting Information Available. Methods of expression and purification of proteins, measurement of mature chromophore concentration, evaluation of $\Delta\mu$ from 2PA spectra, multi-Gaussian fits of 1PA and 2PA spectra, estimation of errors in $\Delta\mu$ due to different methods of fitting of 2PA spectra, estimation of statistical reliability of parabolic fitting curves and the errors in chromophore parameters. This information is available free of charge via Internet at <http://pubs.acs.org>.

variations, there are no direct measurements of the intrinsic electric field deep in the protein structure, and the physical picture of the effect remains unclear.

Here we introduce a new all-optical approach that enables us to directly measure the electric field inside a protein and rationalize the puzzling color changes in the Fruit series. Suppose the permanent dipole moment in the chromophore excited state (μ_e) is different from that in the ground state (μ_g), resulting in non-zero value of $\Delta\mu = \mu_e - \mu_g$. Then, provided that there is a local electric field inside the protein matrix, the internal Stark effect may produce a shift of the chromophore absorption maximum relative to the value in the absence of a field. Note that since the internal field originates from the protein beta barrel, its direction is fixed with respect to chromophore orientation. Therefore, the Stark shift will be the same for all protein particles regardless of their macroscopic (e. g. isotropic) arrangement in the sample. The amplitude, direction, and gradient of the field could be tuned by changing the charge on certain residues or altering the hydrogen-bond and salt bridge network in the chromophore surrounding. Furthermore, if the polarizability tensor of the chromophore α has substantially large components, then a strong local electric field E will create an additional, induced, permanent dipole moment on the chromophore. The intrinsic (vacuum) dipole moment difference between the excited and the ground states $\Delta\mu_0$ is superimposed with the induced value, $\Delta\mu_{ind}$ ($\Delta\mu_{ind} = \Delta\alpha f_S E$, where $\Delta\alpha$ is the difference of polarizabilities in these states and f_S is the static local field factor). If the absolute values of $\Delta\mu_{ind}$ are rather large, then the absorption frequency will depend quadratically on the electric field (second order, or quadratic Stark effect).

A first order Stark effect has been observed in some FPs (16–18). Using Stark spectroscopy, Boxer, Moerner, and co-authors have found that in RFPs, $\Delta\mu = |\Delta\mu| = (5 - 7 D)/f_S$, and that a uniform external electric field of the order of 1 MV/cm only produces a linear Stark effect (17,19). This field strength was probably not large enough to create any additional induced dipole moment difference of the absolute value similar to $\Delta\mu$. The internal electric field of a protein may be much stronger than the typical values of external field that can be potentially applied, i.e., up to 10 – 80 MV/cm (19–21). In these varying field strengths corresponding to a series of different proteins, Callis and co-workers have shown that the tryptophan fluorescence peak exerts a virtually linear internal Stark shift (20) probably because of relatively small $\Delta\alpha$ components of tryptophan. On the other hand, a large change of induced dipole moment due to protein environment has been shown for spheroidene in photosynthetic antenna complex (22), which is consistent with a quadratic Stark effect.

The strong internal electric fields necessary to produce quadratic Stark effect in proteins were previously predicted theoretically (20,23) or estimated from the hole burning experiments by using quantum-mechanically assessed values of $\Delta\alpha$ (17,19). However, full-experimental access to these fields has yet to be realized. We have recently shown (24) that the value of $\Delta\mu$ in the GFP chromophore can be determined experimentally by measuring the 2PA cross section in the maximum of the pure electronic (0–0) transition. In combination with the absorption peak shift, $\Delta\mu$ can then be used as an accurate metric of the local internal field within a protein. Here, we apply this approach to a series of red FPs, derived from DsRed.

Figure 1 shows the 2PA spectra of the Fruit series. DsRed2 (25), mRFP (26) and mCherry at pH11.2 (10) are also included. All the proteins show the 2PA band in the region 1000 – 1200 nm which corresponds to the first electronic, $S_0 \rightarrow S_1$ transition (27). The maximum of the 2PA band can be assigned to a vibronic, 0–1, transition (27). The weaker 2PA 0–0 transition appears as a low-frequency shoulder. To obtain the $\sigma_2(0-0)$ value, we fit the 2PA spectrum in the main part of the $S_0 \rightarrow S_1$ band with a sum of two Gaussians. The fitting was performed with the fixed frequency of the 0–0 transition, marked by an arrow and set equal to the one-photon absorption 0–0 transition frequency, and with variable frequency of the vibronic, 0–1 transition. (Several other methods of multi-Gaussian fits were also explored and gave similar

results, see Supplementary Materials.) The $\Delta\mu$ values for all the proteins were obtained within the two-level approximation of 2PA transition, by using the $\sigma_2(0-0)$ values and maximum extinction coefficients, $\epsilon(0-0)$ (28), as follows (See Supplementary Materials for more details):

$$|\Delta\mu| = \left(\frac{5}{4(1+2\cos^2\gamma)} \frac{hc^2 N_A}{\pi 10^3 \ln 10} \frac{n}{f_{opt}^2} \frac{\bar{\nu}_{0-0}}{\epsilon(0-0)} \sigma_2(0-0) \right)^{1/2}, \quad (1)$$

where h is the Planck's constant, c is the speed of light, N_A is the Avogadro number, n is the refractive index of the medium ($n = 1.33$), $\bar{\nu}_{0-0}$ is the central frequency of the 0-0 transition (in cm^{-1}), γ is the angle between $\Delta\mu$ and transition dipole moment μ , f_{opt} is the local field factor at optical frequency. The results are presented in Table 1.

The change of permanent dipole moment was previously measured for some of these red fluorescent proteins using Stark spectroscopy (17,18). For wt-DsRed, the authors found $|\Delta\mu| f_S = 7.0$ D (17), for mRFP: $|\Delta\mu| f_S = 6 \pm 1$ D (18), and for mPlum $|\Delta\mu| f_S = 5 \pm 1$ D (18). If we compare our results for DsRed2 ($|\Delta\mu| = 3.55$ D), mRFP ($|\Delta\mu| = 2.75$ D), and mPlum ($|\Delta\mu| = 2.55$ D) with these values (29), we notice that for all three proteins $|\Delta\mu| f_S$ values are systematically larger by a factor of 2 than $|\Delta\mu|$, and therefore, $f_S \approx 2$. Assuming that in protein solutions f_S value is determined mostly by the local protein matrix around the chromophore, but not the bulk solvent (30), and using Lorentz model, $f_S = (\epsilon + 2)/3$, we find that the interprotein effective dielectric constant, $\epsilon = 4$. This value falls well within the limits, usually accepted for modeling protein interiors, $\epsilon = 2 - 4$ (31).

Inspection of Table 1 also reveals that $\Delta\mu$ increases dramatically in the series, starting from 1 D in mTangerine and reaching 4 D in mBanana. This increase implies that there must be sizable variations of induced dipole moment from one FP to the next, which are directly related to the large changes of $\mathbf{E}$.

Let ν_0 be the pure electronic transition frequency of the chromophore in vacuum (i.e. for a hypothetical case of the chromophore experiencing no electrostatic interactions with the surrounding); $\Delta\mu_0$ be the difference of vectors of permanent dipole moments in the excited and the ground states in vacuum; and $\Delta\alpha_0$ be the difference between the corresponding tensors of polarizability in vacuum. Let us define an effective electric field that is created by all types of electrostatic interactions (long-range Coulomb, short range dipole-dipole, hydrogen-bonding, etc.) at the chromophore site as $\mathbf{E}$, varying from one mutant to another. Then the absorption transition energy of a chromophore in protein environment can be presented in point dipole approximation as (see e.g. (32)):

$$h\nu = h\nu_0 - f_S \Delta\mu \cdot \mathbf{E}, \quad (2)$$

where

$$\Delta\mu = \Delta\mu_0 + \frac{1}{2} \Delta\alpha_0 f_S \mathbf{E}, \quad (3)$$

The second term in (3) is, by definition, one-half of the induced dipole moment difference. At this point, we can substitute the unknown electric field in (2) with the $\Delta\mu$ by re-arranging Eq. (3) (cf. ref. (33)):

$$\mathbf{E} = 2(f_S \Delta\alpha_0)^{-1} (\Delta\mu - \Delta\mu_0), \quad (4)$$

where $(\Delta\alpha_0)^{-1}$ is the matrix inverse to $\Delta\alpha_0$. This gives:

$$h\nu = h\nu_0 - 2 \Delta\mu \bullet (\Delta\alpha_0)^{-1} (\Delta\mu - \Delta\mu_0). \quad (5)$$

Now suppose that $\Delta\alpha_0$ has a major component only along the direction of $\Delta\mu_0$, i.e. $\Delta\alpha_0$ can be considered as a scalar $\Delta\alpha_0$. This assumption is quite common for charge-transfer chromophores with $\Delta\mu_0 \gtrsim 1$ D (33,34 and references therein), is also supported by our data on 2PA isotropic polarization ratio presented in SI, and also justified by recent quantum-chemical calculations on a model red chromophore (35), implying that $\Delta\mu_0$, $\Delta\mu_{\text{ind}}$ and $\Delta\mu$ vectors are either parallel or antiparallel to each other. Taking this into account, and converting frequency into wavenumbers, we obtain:

$$\bar{\nu} = \bar{\nu}_0 + \frac{2}{hc\Delta\alpha_0} (\Delta\mu_0 \bullet \Delta\mu) - \frac{2}{hc\Delta\alpha_0} |\Delta\mu|^2. \quad (6)$$

This equation is an alternative representation (with respect to standard (2) and (3)) of the second order internal Stark effect.

In Figure 2 we plot the frequency of the 1PA 0–0 transition of the Fruit FPs series as a function of $\Delta\mu$. The dependence fits very well with a parabola: $y = A + Bx + Cx^2$. Comparing the best-fit coefficients with their expressions in (6), we obtain the following parameters of the red chromophore: $\bar{\nu}_0 = (19350 \pm 120) \text{ cm}^{-1}$, $\Delta\mu_0 = (4.49 \pm 0.27) \text{ D}$ (where $\Delta\mu_0$ is the projection of $\Delta\mu_0$ on $\Delta\mu$), and $\Delta\alpha_0 = (-20.6 \pm 0.8) \text{ \AA}^3$. Nifosi et al. (8) calculated the vacuum transition frequency of DsRed chromophore, $\bar{\nu}_0 = 19030 \text{ cm}^{-1}$, using TDDFT with GB-B3LYP functional, and, vacuum $\Delta\mu_0 = 4.69 \text{ D}$ with BLYP functional. Both our experimental values correlate perfectly with the theoretical results. The $\Delta\alpha_0$ tensor was calculated in (35) for a model 4-hydroxy-benzylidene-1-methyl-2-propenyl-imidazolinone red chromophore, and it was found that $\Delta\alpha_0$ has a main component along one selected axis, equal to $\Delta\alpha_0 = -15 \text{ \AA}^3$. This result is similar to our experimental value both in amplitude and sign. These rather good correlations between experimental and calculated values help to justify our initial assumption on the applicability of two-level approximation for the 2PA transition.

Several important conclusions stem from Fig. 2. First, one can see that there is a limiting minimum absorption frequency, equal to $\bar{\nu} = 16900 \text{ cm}^{-1}$ (longest possible wavelength of 0–0 transition, 592 nm), that virtually corresponds to both mCherry and mPlum. In other words, no mutations in the barrel structure can further red-shift this chromophore absorption. Second, we find that $\Delta\alpha_0$ is negative, which means that the polarizability of the chromophore in the excited state is less than in the ground state. The sign of $\Delta\alpha_0$ defines the direction of $\Delta\mu_{\text{ind}}$ if the direction of electric field is given. Also, the negative sign of the linear term in the parabolic fit in Fig. 2 implies that the dot product $(\Delta\mu_0 \bullet \Delta\mu)$ is positive, and therefore vector $\Delta\mu$ is co-directed with vector $\Delta\mu_0$. But since in the studied region $|\Delta\mu| < |\Delta\mu_0|$, we conclude that $\Delta\mu_{\text{ind}}$ is directed oppositely to $\Delta\mu_0$.

For the practical reasons, if one would like to further increase $\Delta\mu$ (e.g. for enhancing two-photon absorption or for improving the sensitivity of probing an electric field), one would need to create a mutant where the internal electric field vector will be oriented in the opposite direction to the one found in the Fruits. Such change will, however, inevitably cause a blue shift of the absorption maximum.

Knowing $\Delta\mu_0$ and $\Delta\alpha_0$, and using $\Delta\mu$ values presented in Table 1, we can now estimate, using (3), the effective local field at the chromophore site $f_S E$ (more exactly - projection of the field

vector on $\Delta\mu_0$). The obtained values of the field strength vary from 10 to 100 MV/cm (Table 1), which is well in the range of previous theoretical estimations for myoglobin (19), cytochrome c (21), calmodulin (20), cone opsins (37), and other proteins (20). To our knowledge, this is the first time that internal field of a protein is measured experimentally. It is also instructional to compare these values to other electric fields encountered in biological systems. For example, the field change across the membrane of a neuron during the action potential is about two orders of magnitude smaller, ~ 0.3 MV/cm. On the other hand, the field binding an electron to the GFP chromophore (corresponding to ionization potential) is still about 1 – 2 orders of magnitude higher (38) than the intraprotein field.

In conclusion, the differences in hue in the Fruit series of red FPs are caused by a quadratic Stark effect exerted on the chromophore by the protein environment. The knowledge of the mechanism of color tuning in FPs, and direction of permanent dipole difference, will facilitate rational design of new mutants with desired optical properties, including improved field sensitivity, two-photon brightness, or absorption/emission wavelength. The internal Stark effect likely tunes the absorption of other FPs and chromoproteins as well. For example, electrostatic interactions in the human color opsins are thought to tune the absorption properties of retinal (39). The experimental approach and physical model proposed here may also provide useful insights into other protein realms, such as enzymatic activity and folding processes. Other all-optical methods interrogating two-photon transitions, such as resonance hyper-Raman and hyper-Rayleigh spectroscopies (40) can potentially provide an access to $\Delta\mu$ (and, therefore, internal electric fields) if a weakly- or non-fluorescing chromophore is used as a probe.

It appears that the striking beauty of a coral reef, both in the variety of colors it contains and the way we perceive them, involves the effects of strong electric fields occurring within a nanoscopic protein environment: both the opsin proteins in the eye of the beholder and the beta-barrel of the fluorescent proteins shape perception through Stark effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Two-photon absorption spectra of a series of red FPs in the region of the first electronic transition. 2PA cross section σ_2 (in GM, $1\text{GM} = 10^{-50} \text{ cm}^4 \text{ s}$) is plotted versus transition frequency (equal to twice the laser photon frequency). Laser wavelength used for excitation is shown as a top x -axis. The fit with a sum of two Gaussians is shown with continuous line. Individual Gaussians are shown by dashed lines. An arrow depicts the 0–0 1PA transition frequency which was kept fixed upon fitting of 2PA spectra.

Figure 2.

Dependence of the pure electronic $S_0 \rightarrow S_1$ transition frequency (which is very close to 1PA maximum) on permanent dipole moment difference between S_1 and S_0 states for a series of red FPs. Top x -axis shows the projection of effective electric field on the direction of $\Delta\mu_0$. f_s is the local field factor. Continuous line shows the best fit with second order polynomial: $y = A + Bx + Cx^2$ with the coefficients $A = 19350 \pm 116 \text{ cm}^{-1}$, $B = -2180 \pm 100 \text{ cm}^{-1} \text{ D}^{-1}$, $C = 486 \pm 20 \text{ cm}^{-1} \text{ D}^{-2}$. The inset shows the chromophore structure. Note that the crystal structures, available for DsRed (9), mStrawberry (10), mCherry (10), mPlum (11), and mBanana (12), show that the chromophore is the same for these five proteins and contains the acylimine group. For the remaining four proteins, there are strong indications that the chromophore structure is the same as shown. It is known that the originally produced acylimine tail of the DsRed-type chromophore can be attacked by either $-\text{OH}$ group of Thr66 or $-\text{SH}$ group of Cys66, to produce new chromophores in mOrange (10) and mKO (34), respectively. For this reaction to occur Glu215 should be deprotonated and, in the case of Cys66, position

70 should be occupied by Arg, not Lys. mRFP is a product of DsRed with no mutations in close proximity to the chromophore, including position 66. mRFP is also a progenitor for mCherry and mStrawberry, which both have the same chromophore as DsRed. tdTomato and mCherry (at pH11.2) have Q66M mutation, which should not have nucleophilic activity. mTangerine, similarly to mBanana holding intact acylimine tail, possesses Q66C mutation, but has Lys in position 70.

Table 1

Optical properties of a series of red fluorescent proteins. Unless otherwise stated, all the data are presented for pH 8 buffer solutions. $\nu(0-0)$ is the maximum of pure electronic $S_0 \rightarrow S_1$ transition; $\epsilon_{\max}(0-0)$ is the extinction coefficient at this frequency; $\sigma_2(0-0)$ is the two-photon absorption cross section at $\nu(0-0)$, obtained as described in the text and measured in GM ($1 \text{ GM} = 10^{-50} \text{ cm}^4 \text{ s}$); $\Delta\mu$ is the absolute value of the difference between the permanent dipole moment in the excited (S_1) and the ground (S_0) state, measured in Debye (D); $\Delta\mu_{\text{ind}}$ is the projection of induced dipole moment difference (between S_1 and S_0) on $\Delta\mu_0$. $f_S E$ is an effective local electric field projection on $\Delta\mu_0$. f_S is the local field factor.

Protein	$\nu(0-0)$ (cm^{-1})	$\epsilon_{\max}(0-0)$ ($10^3 \text{ M}^{-1} \text{ cm}^{-1}$)	$\sigma_2(0-0)$ (GM)	$\Delta\mu$ (D)	$\Delta\mu_{\text{ind}}$ (D)	$f_S E$ (MV/cm)
mTangerine	17634	33.5	1.1	1.0	-7.0	102
mStrawberry	17295	40.8	2.7	1.41	-6.2	90
mCherry	16925	61.8	11	2.22	-4.5	66
mPlum	16882	37.5	9.0	2.55	-3.9	57
mRFP	17006	54.2	15	2.75	-3.5	51
mCherry pH 11.2	17685	51.7	22	3.48	-2.0	29
DsRed2	17760	77.1	34	3.55	-1.9	28
tdTomato	17950	58.7	28	3.73	-1.5	22
mBanana	18457	64.1	40	4.04	-0.9	13