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Probing the Decay Coordinate of the Green Fluorescent Protein: Arrest of Cis−Trans Isomerization by the Protein Significantly Narrows the Fluorescence Spectra

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Abstract: The fluorescence spectra of the wild-type green fluorescence protein (wt-GFP) and the anionic form of *p*-hydroxybenzylidenedimethylimidazolone (*p*-HBDI), which models the protein chromophore, were obtained in the 80−300 K temperature range in glycerol/water solvent. The protein spectra have pronounced and well-resolved vibronic structure, at least at lower temperatures. In contrast, the chromophore spectra are very broad and structureless even at the lowest temperatures. Analysis of the spectra shows that the experimentally observed red-shift of the protein spectrum upon heating is apparently caused by quadratic vibronic coupling of the torsional deformation (TD) of the phenyl single bond of the chromophore to the electronic transition. The broad spectra of the chromophore manifest the contribution of different conformations in the glycerol/water solvent. In particular, the lowest-temperature spectrum reflects the distribution over the same TD coordinate in the excited electronic state, which essentially contributes to the asymmetry of the spectrum. Upon heating, motion along this coordinate leads to a configuration from which the radiationless transition takes place. This narrows the distribution along the TD coordinate, causing a more symmetric fluorescence spectrum. We were able to reconstruct the broad, structureless fluorescence spectra of *p*-HBDI in glycerol/water solutions at various temperatures by convoluting the original wt-GFP spectra with the function describing the distribution of the transition energies of the *p*-HBDI chromophore. Thus, both the fluorescence broadening and increase in radiationless transition upon removal of the protein chromophore to bulk solvent are consistent with decay by a barrierless TD of the phenyl single bond.

Introduction

The green fluorescence protein (GFP) is intensively used to study very diverse problems in molecular biology, medicine, biochemistry, and cell biology.[1,2] Such usage takes advantage of the presence of an internal chromophore, which generates a very effective and intense fluorescence. Nevertheless, investigations of a model chromophore as well as certain GFP mutants in different solvents[3−7] reveal a strong disconnect between the properties of the protein and the intact chromophore. For instance, the fluorescence of the free chromophore is much less efficient and strongly depends on the temperature: the higher the temperature, the less effective the fluorescence.[8] These observations suggest that in the model chromophores, an efficient radiationless transition along a coordinate invoking strong conformational rearrangement of the molecule and displacing only a small solvent volume[8] takes place. This rearrangement is arrested in GFP by the rigid protein barrel, making excited-state proton transfer (ESPT) from the chromophore to the adjacent amino acids and bound water molecules possible. As a result, a strong green fluorescence from the anion is observed.[9] Most probably such relaxation in the model compound proceeds down from the Franck−Condon (FC) point to a nonfluorescent twisted intermediate (TI1 or TI2) along torsional deformation (TD) of the phenyl single bond and then to the conical intersection (CI) of the chromophore[10−12] along another more rigid coordinate.[13−15] Nevertheless, despite in-

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tensive study of the fluorescence of GFP, of its mutants, and of the model chromophores at ambient and low temperatures,[3−7,13−19] the contribution of the vibronic coupling of the electronic transition to the TD displacement into the fluorescence band shape still awaits clarification.

In this paper, we report a systematic analysis of the temperature dependence of the fluorescence spectra of wild-type GFP (wt-GFP) and of its model chromophore, *p*-hydroxybenzylidene-dimethylimidazolone (*p*-HBDI). The temperature dependence of the fluorescence spectra of *p*-HBDI, its derivatives, and several GFP mutants has been reported recently.[3,20,21] No significant temperature dependence of the fluorescence quantum yield of wt-GFP was reported, which provides evidence for an almost barrierless ESPT, but a narrowing of the emission lines and development of vibronic structure was observed.[3] Interestingly, the emission of various GFP mutants exhibited a somewhat stronger temperature dependence,[20] and the ESPT in these systems was found to be activated with a ΔE_{act} of 15−30 kJ/mol. In contrast, the emission of the free chromophores in bulk solvents in both neutral and anionic forms demonstrated significant temperature dependence. Upon cooling to 77 K, the fluorescence quantum yield increased by several orders of magnitude, but no spectral shift or broadening was reported.[21] In this work, we present a spectral analysis that allows us to expose the peculiarities of the vibronic interactions in the chromophore and, in particular, the effect of the TD displacements on the band shape of both the wt-GFP and *p*-HBDI spectra and to draw conclusions about the mechanism of internal conversion in this system.

Material and Methods

For excitation, we used a cavity-dumped mode-locked Ti/sapphire femtosecond laser (Mira Coherent), which provides short (80 fs 500 kHz) pulses of variable repetition rate. This was done for the sake of comparison with the time-resolved data that will be published in subsequent papers. We used the second harmonic generation frequency over the spectral range of 380−400 nm. The excitation pulse energy was reduced by neutral density filters to about 1 pJ. We checked the absorption of the sample absorption prior to and after time-resolved measurements and could not find noticeable changes in the absorption spectra due to sample irradiation. The laser beam was redirected, and the steady-state fluorescence spectra were taken at various temperatures using a CVI SM-240 spectrometer with about 2 nm resolution and also were measured using a FluoroMax-3 spectrofluorometer (Jobin Yvon). The temperature of the irradiated sample was controlled by placing the sample in a liquid-nitrogen cryostat with thermal stability of approximately ±1 K. The irradiated sample was in buffered (pH 7.9) aqueous solution containing about 12% glycerol.

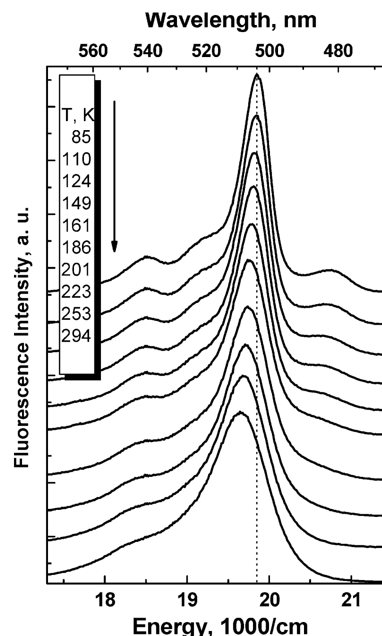

Figure 1. Wt-GFP fluorescence spectra obtained at (from top to bottom) 85, 110, 124, 149, 161, 186, 201, 223, 253, and 294 K. Vertical line shows emission maximum at lowest temperature.

p-HBDI was synthesized according to published procedures.[22] The steady-state emission spectra of the anionic form of *p*-HBDI were measured in glycerol/water solution of composition of 0.4 mole fraction of glycerol in a basic solution of pH 10. We received wt-GFP samples from Prof. S. J. Remington of the Institute of Molecular Biology, University of Oregon at Eugene.

Results

The data on the temperature dependence of the wt-GFP steady-state fluorescence spectra are presented in Figure 1. The ∼480 nm peak observed at the low temperatures corresponds to the fluorescence of the relaxed anionic form of the protein.[3] The low-energy parts ($\lambda \leq 500$ nm) of the spectra, which correspond to the luminescence of the chromophore anion formed by ESPT, are analyzed below. These spectra exhibit a well-resolved vibronic progression, which is clearly seen at lower temperatures. The presence of the vibronic progressions and the thermal broadening of each of their components imply that the excited electronic state is shifted with respect to the ground state along a number of nuclear normal coordinates (Q_i), the difference between force-field constants of these states along these coordinates being negligible.[23] This is the case of the so-called linear electron−vibrational coupling of the electronic transition to the nuclear coordinates of the chromophore or its environment.

We note that the low-temperature wt-GFP spectra contain a strong vibronic satellite at ∼1400 cm^{-1}. This energy is close to the 1450 cm^{-1} frequency of the vibration that corresponds to the displacement responsible for the transformation of the FC conformation of the excited electronic state to the fluorescent state (FS) conformation and, consequently, is strongly coupled to electronic transition, as noted by Olivucci et al.[14]

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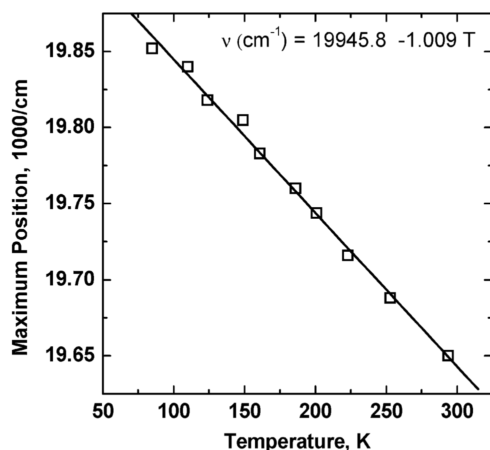

Figure 2. Temperature dependence of the main peak position of wt-GFP (□) and its linear fit (line).

Upon heating, the spectrum shifts to the red, this shift being linearly dependent on the temperature (Figure 2). Such behavior is a manifestation of the so-called quadratic electronic−vibrational coupling:[24,25] the presence of normal coordinates (q_i), along which the force-field constants of the ground electronic state differ from those of the excited one.[24,25] Moreover, the fact that the whole spectrum rigidly shifts implies[24] that the displacements along the q coordinates are characterized by considerably smaller force-field constants than those along the Q coordinates.

It follows from this consideration that the electronic transition of the chromophore is vibronically coupled to a number of the nuclear displacements, with a linear or quadratic form depending on the nature of the displacement. We note that the narrow bands and the presence of the resolved vibronic structure suggest that the chromophore is locked into one conformation and its surroundings are well structured, because a disordered environment and/or a distribution of the chromophore over different conformations are expected to lead to a general broadening of the bands.

The latter kind of spectrum is manifested by the anionic form of the isolated chromophore, *p*-HBDI, stripped of the protein environment and surrounded by glycerol/water solvent at pH 10. Such a sample has much broader steady-state spectra at all temperatures, lacking any vibronic structure both in the glassy and liquid states (glass−liquid transition temperature, $T_c \approx 180$ K), see Figure 3.

This strong broadening can stem from an electrostatic interaction of the chromophores with the solvent molecules or from a distribution of the chromophore over different conformations in the excited state stabilized by the solvent. In the wt-GFP matrix, the chromophore conformation is constrained by the very rigid protein matrix, which makes such a distribution very narrow.

Bearing in mind that the wt-GFP spectra are much narrower than those of the free chromophore in solution, we assume that the protein spectrum may be considered as the spectrum of "free chromophore in vacuum" locked into one of its conformations, $F(\omega)$. We take into account that the chromophore spectral broadening is controlled by the two types of the geometry variations (of the chromophore or the solvent molecules), which are coupled to the electronic transition linearly (Q_i) and quadratically (q_i). Assuming that Q_i and q_i vary according to the normal distribution, we reconstruct the free-chromophore spectrum in the solution from the protein spectrum, obtained at the same temperature.

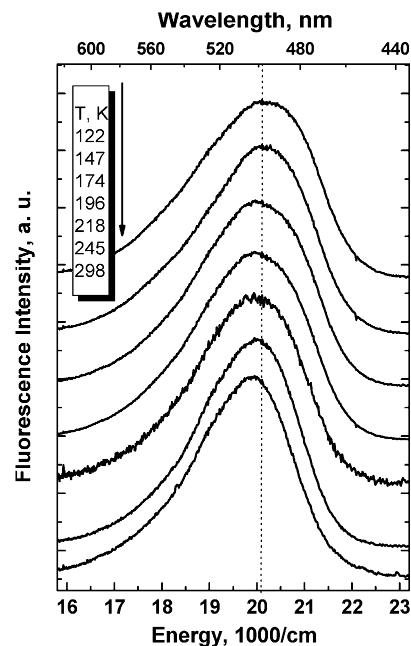

Figure 3. Temperature dependence of the shape of the fluorescence spectra of anionic form of *p*-HBDI obtained at pH 10 at (from top to bottom) 122, 147, 174, 196, 218, 245, and 298 K. Band integral intensities are normalized to 1. Vertical line shows emission maximum at lowest temperature.

In the protein and in *p*-HBDI, the anionic excited electronic state is populated in different ways: in *p*-HBDI, it is excited directly at pH 10, because the lifetime of the substrate is too slow to allow for proton transfer from its neutral form; in wt-GFP, the neutral (protonated) species is excited first and then loses one proton and relaxes to the lowest excited electronic state of the anion. In principle, the fluorescent states of the chromophore in the protein and in solution could be different. However, the known data suggest that these FSs are the same. Indeed, the ground-state structures of the protein chromophore and *p*-HBDI are nearly planar.[26] The geometry of the latter weakly changes upon the excitation, and the FS and FC points have the same electronic nature and very close geometries.[13] The position of the fluorescence peak of the free chromophore is very close to the position of the fluorescence peak of the anionic form of the chromophore in the protein (see Figures 1 and 3). In the latter case, at very low temperatures, two types of the anionic forms of the chromophore exist in wt-GFP ($\lambda_{max} \approx 502$ and 510 nm),[27] which apparently correspond to a slightly different protein environment for the chromophore. The spectra of wt-GFP and of its anion have the same shape and are shifted by the nonrelaxed protein environment.[3,16,17,28] These facts suggest that the FSs of wt-GFP and the model compound have the same electronic nature and that the steady-state spectra obtained in this work are not affected by the mode of their population.

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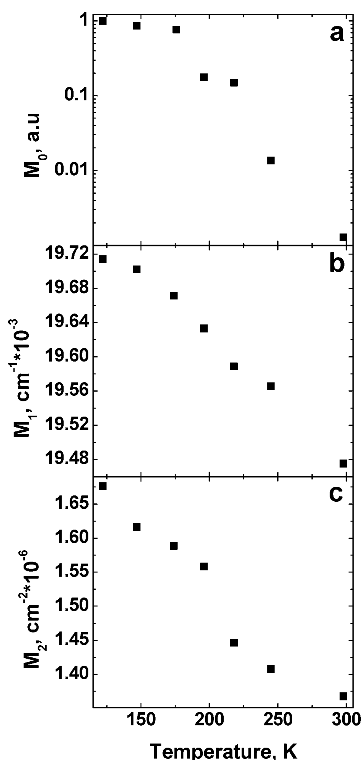

Figure 4. Temperature dependence of the first three moments of the *p*-HBDI anion flourescence spectra.

The integrated intensity of the wt-GFP fluorescence is largely unaffected by the temperature change, whereas each component of the wt-GFP spectrum is broadened by the temperature increase (see Figure 1). In contrast, the integrated intensity (M_0) of the *p*-HBDI spectra is strongly weakened (see Figure 4a)[4,8,23] and the width ($\sim\sqrt{M_2}$) of the *p*-HBDI band is reduced (Figure 4c) upon heating. Moreover, it is clear from Figure 3 that heating reduces the asymmetry of the *p*-HBDI band. This very different and unusual temperature dependence of *p*-HBDI is presumably caused by radiationless decay of the fluorescent state of the former, a transition that becomes more effective at higher temperatures.

To check this assumption and to reveal the nature of the coordinate of the radiationless transition, reconstruction was performed for the spectra obtained at two temperatures: at 122 K, at which the radiationless transition in *p*-HBDI was very weak and therefore hardly contributed to the fluorescence band shape and intensity, and at 253 K, at which the radiationless transition strongly affected the spectrum, reducing its intensity by about 2 orders of magnitude (see Figure 4a). We used the 124 and 245 K spectra of wt-GFP, respectively. The use of slightly different temperatures for the wt-GFP do not affect the conclusions. In the case of the 124 K spectrum of wt-GFP, the ~480 nm peak was modeled by a Gaussian and subtracted from the protein spectrum.

We also assumed that, at both temperatures, the distribution of the free chromophore in FS along all the coordinates was normal. This assumption was straightforward at low temperature, where the FE radiationless decay was very weak, hardly affecting the fluorescence intensity (see Figure 4). At higher temperature, we suggest that the effective FE → TI radiationless transition along the transition coordinate made the distribution in this direction negligibly narrow, with the distributions over other coordinates being weakly affected.

Initially, we attempted to reconstruct the lower-temperature spectrum of *p*-HBDI. We tried to include a distribution of the molecules over both linearly and quadratically coupled coordinates to reconstruct the spectrum of the model compound. To do this, first we neglected the distribution over the quadratically coupled to the transition q coordinate and described the contribution of the Q_i distribution by a distribution of only one effective Q coordinate with width σ_{0Q} and center Q_0.

$$P_Q(Q) = \frac{1}{\sqrt{2\pi}\sigma_{0Q}} \exp\left[-\frac{(Q-Q_0)^2}{2\sigma_{0Q}^{\ 2}}\right] \tag{1}$$

Here, Q_0 takes into account a possibility that the center of the distribution of the Q coordinate differs from its value in the protein. In the case of the linear coupling

$$\Delta E = BQ + C \tag{2}$$

the normal distribution in Q, $P_Q(Q)$, would cause a normal distribution also in the energy space, $P_Q(\omega)$

$$P_Q(\omega) = \frac{1}{\sqrt{2\pi}\sigma_Q} \exp\left[-\frac{(\omega-\omega_Q)^2}{2\sigma_Q^{\ 2}}\right] \tag{3}$$

where

$$\sigma_Q = B\sigma_{0Q}$$

and

$$\omega_Q = BQ_0 + C \tag{4}$$

The broadening of the bands of the free chromophore could be obtained by the convolution

$$\Phi(\omega) = F(\omega) \otimes P_Q(\omega) \tag{5}$$

Using eq 5, the protein spectra, and constants ω_L and σ_Q as the fitting parameters, we attempted to reconstruct the shape of the free-chromophore spectra. As seen in Figure 5, at the lower temperature (122 K), this approach gave an asymmetric curve, which did not adequately fit the *p*-HBDI spectrum.

Next, we took into account only the distribution over the quadratically coupled coordinate q_i, which was simulated by the normal distribution of one effective q coordinate with the center of the distribution q_0 and its width $\sigma_{0q}(T)$. In this case, the energy gap ΔE of the transition depends quadratically on q.

$$\Delta E = aq^2 + bq + c \tag{6}$$

Assuming that the force-field constant of the ground electronic state is larger than that of the excited state ($a > 0$), one easily

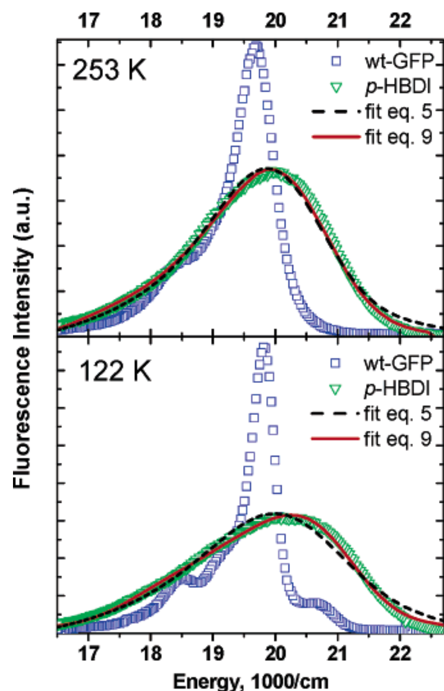

Figure 5. Reconstruction of the model spectra (▽) by utilizing the protein spectra (□), eq 5 (dashed curve), and eq 9 (solid curve)

obtains[29] that the normal distribution of the q coordinate causes the following distribution in the energy space

$$P_q(\omega) = \frac{1}{2\sigma_q\sqrt{2\pi(\omega - \omega_q)}}\left\{\exp\left[-\frac{(\sqrt{\omega - \omega_q} - d)^2}{2{\sigma_q}^2}\right] + \exp\left[-\frac{(\sqrt{\omega - \omega_q} + d)^2}{2{\sigma_q}^2}\right]\right\} \quad (7)$$

where

$$\omega_q = c - \frac{b^2}{4a}$$

$$d = \frac{b}{\sqrt{a}} + 2\sqrt{a}q_0$$

and

$$\sigma_q = \sigma_{0q}\sqrt{a} \quad (8)$$

The attempt to fit the p-HBDI spectra taking into account only the distribution of q also failed, giving a very different shape of the model compound spectrum (not presented on Figure 5) and indicating that the distributions along both Q and q must be taken into account. These distributions led to the broadening of the free-chromophore spectrum $F(\omega)$, according to the expression

$$\Phi(\omega) = F(\omega) \otimes P_Q(\omega) \otimes P_q(\omega) \quad (9)$$

Expression 9 was used to fit the 122 K p-HBDI spectrum utilizing the 124 K protein spectrum. The results of the fitting procedure are presented in Figure 5, using the fitting parameters: $\sigma_q = 1.25$ cm^{-1}, $d = 12$ cm^{-1}, $\sigma_Q = 662$ cm^{-1}, and $\omega_Q = 1120$ cm^{-1} (ω_q was assumed to equal zero to reduce the number of parameters). The weak discrepancy between the modeled and experimental spectra on the blue side was presumably caused by the ambiguous procedure of the subtraction of the 480 nm band, corresponding to the fluorescence of the relaxed anionic form of wt-GFP. It follows from eqs 4 and 8 that the σ_q and σ_Q values are controlled not only by the widths of the q and Q distributions but also by the strengths of the linear (B) and quadratic (a) couplings. Therefore, the much bigger value of σ_Q compared to that of σ_q does not imply that the Q coordinate is distributed more strongly than the q one. Note also that it was assumed in the procedure under consideration that parameters describing the relationship between the structure of the chromophore and the energy of the electronic transition (a, b, c, B, and C in eqs 2 and 6) are temperature independent, whereas the distribution parameters (σ_{0Q}, Q_0, σ_{0q}, and q_0, eq 1) can depend on the sample temperature.

We next turned to the reconstruction of the higher temperature p-HBDI spectrum. First we assumed that there is a normal distribution over the q coordinate only, but as in the low temperature case, the resulting curve was very different from the experimentally observed spectrum. Then we assumed that only a distribution of the Q coordinate takes place and used eq 5 to fit the p-HBDI spectrum. It can be seen in Figure 5 that the quality of the reconstruction of the latter at 253 K is acceptable ($\sigma_Q = 710$ cm^{-1} and $\omega_Q = 386$ cm^{-1}). Inclusion into consideration of the distribution over both the Q and q coordinates very weakly improved the quality of the fit, showing that at the higher temperature, the distribution of the q coordinate hardly affects the band shape.

This result showed that on the one hand, the distribution of the quadratically coupled coordinate in FS is strongly narrowed due to the FS → TI radiationless transition and, on the other hand, even at higher temperatures, a significant distribution of the model compound over the linearly coupled coordinate(s) still persists in FS.

To check if the specific protein spectra are important for the successful reconstruction of the free-chromophore spectra, we attempted to fit the p-HBDI spectra in solution by a convolution of a Gaussian distribution (eq 3) with quadratic (eq 7) distributions

$$\Phi(\omega) = P_Q(\omega) \otimes P_q(\omega) \quad (10)$$

The fitting curve has a very different shape (not shown in Figure 5) and cannot fit the experimentally observed p-HBDI spectrum; this clearly shows that taking into account the protein spectra is necessary for a successful interpretation of the p-HBDI spectra.

Discussion

It follows from the examination of the wt-GFP spectra that there are two kinds of nuclear normal coordinates significantly contributing to the temperature dependence of the spectral shape. The first type corresponds to normal coordinates, which are coupled to the optical transition linearly, and causes the appearance of the vibronic progressions. The second kind of

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coordinates are coupled to the optical transition quadratically and cause the red-shift of the spectrum as a whole upon the heating, because the force-field constants of the normal coordinates of the second type in the excited state are lower than in the ground state. It is important to note that the observed modes belong not to intermolecular hydrogen bonds with solvent but to intramolecular vibrations, because ultrafast deactivation of *p*-HBDI was observed also in nonprotic solvents.[8,30] Moreover, the parameters obtained as results of the fitting procedures characterize the distributions of the chromophore along different normal coordinates and vibrations by themselves. Therefore, they cannot be compared directly to the IR or resonance Raman vibrational frequencies of *p*-HBDI and do not provide insight into the volume conserving "hula twist" proposed as the deactivation pathway in viscous solvents.[26]

The nature of the coordinate that mainly contributes to the second type of the normal coordinate can be elucidated by comparing the fluorescence spectra of the wt-GFP and *p*-HBDI. First, we note that there is a coordinate along which the energy gap between the excited and ground electronic states responsible for fluorescence has a nonlinear[6,10−12,14−16] and, to a first approximation, quadratic dependence; this coordinate corresponds to the TD displacement of the chromophore. The force-field constant of the corresponding displacement is low; therefore, this coordinate can be considered as a candidate for a role of the quadratically coupled coordinate coupled to the fluorescence transition under consideration.

Second, it was shown recently[14,15] that the FS conformation very effectively relaxes to the nonfluorescence TI1 conformation along the TD coordinate of the chromophore. This implies that there is strong electron−vibrational coupling of the FS excited electronic state to the TD coordinate. Consequently, distribution along this coordinate is expected to strongly affect the band shape of *p*-HBDI.

Third, the free chromophore in a solvent can easily move along the TD coordinate and adopt different conformations depending on the solvent environment, at least at temperatures higher than the temperature of the glass−liquid transition of the solvent, ∼180−200 K; whereas in wt-GFP, the protein barrel causes other conformations to have much higher energy, resulting in fluorescence band narrowing. In other words, the adiabatic potential along this coordinate in the protein is much steeper than in solution. This consideration also supports the assignment of q to the TD coordinate, which is widely distributed in the solvated *p*-HBDI chromophore and is well defined in the protein. However, the distribution parameters (Q, σ_Q, q, and σ_q) do not represent a distribution of some specific coordinate. They are effective parameters describing a convolution of the Gaussian distributions along different coordinates, because a convolution of a number of Gaussians is a Gaussian with effective maximum position and width.

Fourth, it is well-known[3−7,13−15] and is supported by the results of this study that in model compounds such as *p*-HBDI in liquid solution, a radiationless transition between the excited and ground electronic states takes place; the higher the temperature, the more effective this transition.[3−7] The radiationless transition is caused by the motion of the system along the TD coordinate of the model compound [6,10−12,14−16] down to the nonfluorescent TI1 conformation[14] and then to the conical intersection along another much rigid coordinate. In support of this view, in a recent study we have observed that the excited state of the chromophore possesses a substantial twist in the otherwise planar chromophore.[31] This is also supported by a careful study of the fluorescence lifetime behavior of *p*-HBDI in solvents of varying viscosity.[32] Therefore, a FS → TI1 motion of the system is expected to narrow the distribution of the free chromophore in the FS conformation along the TD coordinate and reduce the FS population. Consequently, the higher the temperature, the narrower the distribution of the fluorescent molecules along this TD coordinate and the weaker the expected fluorescence. Thus, the distribution along the TD coordinate is expected to only weakly affect the *p*-HBDI fluorescence spectra at higher temperatures and strongly affect it at lower temperatures in the glassy matrix.

This is exactly what is observed: to obtain the low-temperature (122 K) *p*-HBDI spectrum from the protein spectrum, we must assume that the substantial distribution along q takes place in the former, whereas the more symmetric 253 K spectrum can be reconstructed neglecting this distribution. This implies that at the higher temperature, the distribution of the model chromophores along q is too narrow to notably affect the shape of *p*-HBDI fluorescence. This temperature dependence of the q distribution also correlates with its assignment to the TD coordinate. It is this narrowing in the q distribution that causes very unusual narrowing of the *p*-HBDI fluorescence band upon heating, concomitant with an increase in the excited-state decay rate (see Figure 5).

Fifth, our assumption that the q force-field constant of the ground electronic state is larger than that of the excited state ($a > 0$ in eq 6) agrees with the assignment of q to the TD coordinate. Indeed, the chromophore energy depends on the TD coordinate much steeper in the ground state than in the excited one.[12,14]

The fact that the width of the distribution of the first type coordinate (σ_Q) increases from 662 to 710 cm^{-1} upon heating shows that it is weakly (if at all) affected by the radiationless transition. Probably, it reflects the effect of the distribution of the electric field of the solvent molecules, both static and dynamic: the higher the temperature, the larger the expected amplitude of the motion of these molecules. This explains the increase in σ_Q. It is noteworthy, however, that σ_Q is an effective parameter, and some caution should be taken in interpreting its temperature dependence.

In principle, the asymmetric broadening of the *p*-HBDI spectra can be interpreted assuming that only the linearly coupled coordinates are distributed, but the distribution represents a sum of two or more Gaussians with different centers of distribution. However, this assumption does not appear well founded for the following reasons: First, it is not clear why the conformations of the free chromophore in solution should not be distributed along the quadratically coupled coordinate. Second, it is difficult to understand why the distribution along the linearly coupled coordinate should have multimodal character. Third, even if only the sum of two Gaussians were taken into account, the number of the fitting parameters would be larger than in the model considered in this paper (5 against 4).

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All these facts point to the twisting deformation as the motion along which the fluorescent conformation is depopulated at higher temperatures and, consequently, is responsible for the asymmetry and, partially, for the broadening of the low-temperature *p*-HBDI band. This model agrees with the high-level calculations.[13,14]

Conclusions

The consideration presented above shows that probably the rigid shift of the wt-GFP fluorescence spectrum upon heating is a manifestation of the quadratic coupling of the torsional deformation of the phenyl single bond to the electronic transition of the chromophore in the protein. In the case of the solvated free chromophore, the distribution of the *p*-HBDI molecule along this coordinate causes a very specific shape of the low-temperature fluorescence spectrum, this distribution being drastically narrowed at the higher temperatures because of the radiationless decay along the same coordinate. Additional broadening of the *p*-HBDI spectrum stems from the electrostatic interactions with the solvent molecules. As the temperature is raised, the efficacy of the transition from the fluorescent conformation to the nonfluorescent one and to the conical intersection increases, leading to rapid internal conversion.

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