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Steric and Electronic Effects in Capsule-Confined Green Fluorescent Protein Chromophores

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Supporting Information

ABSTRACT: The turn-on of emission in fluorescent protein chromophores sequestered in an "octaacid" capsule is controlled by stereoelectronic effects described by a linear free energy relationship. The stereochemical effects are governed by both the positions and volumes of the aryl substituents, while the electronic effects, including ortho effects, can be treated with Hammett σ parameters. The use of substituent volumes rather than A values reflects packing of the molecule within the confines of the capsule.

The way in which confinement affects fluorescence presents a remarkable set of challenges in supramolecular photochemistry. We have been investigating the sensitivity of the chromophores derived from fluorescent proteins (FPs) to their environment through studies that mimic the effect of the sequestering β-barrel.[1] Such chromophores are known to exhibit fast internal conversion through two torsional modes, ϕ and τ, that are inhibited in the protein (see Figure 1). This facile internal conversion, which represents a 10^4-fold decrease in fluorescence intensity, makes this chromophore very unusual and provides a unique opportunity to investigate details of excited-state decay in confined environments. For instance, we have discovered that sequestration of these benzylidenemethylimidazolidinones (BMIs) within a deep hydrophobic cavitand, the so-called "octaacid" (OA),[2] mimics the β-barrel and turns on the fluorescence in a way that depends on the substitution at the ortho position.[3] In order to separate the influence of electronic and steric effects on this phenomenon, we now report how the fluorescence turn-on is affected by a number of substituted chromophores that differ in both their steric volumes and electronic effects.

The BMI chromophores were synthesized using previously described methods[4] (see R_1 and R_2 in Table 1) and then exposed to buffered solutions of OA in D_2O. In all cases, complexation of the chromophores[5] was validated by the upfield ^{1}H NMR chemical shifts of the guests relative to the values in CD_3CN solvent, corresponding to placement of protons within the shielding region of the aromatic cavity [see the Supporting Information (SI)]. As seen in our earlier studies,[3,6] the alkyl derivatives experienced strong shielding of both aryl-ring and N-alkyl groups within the cavity (see the SI). At the concentrations used here, with a 1:4 guest/host ratio, no unbound guest could be observed by ^{1}H NMR spectroscopy, and additional fluorescence studies confirmed that the fluorescence was at or near saturation.[7] Notably, CH_3CH_2- as an ortho substituent did not lead to a stable complex.

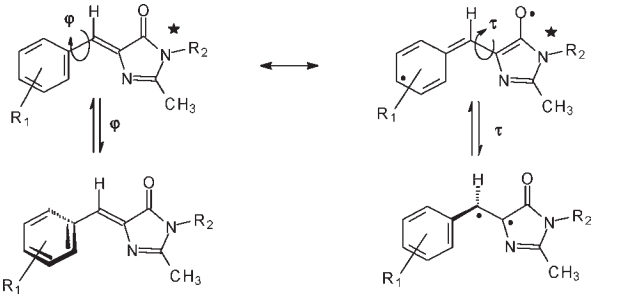

Figure 1. Torsional modes in excited states of BMIs.

We next considered the electronic and steric effects on the excited-state emission using steady-state fluorescence measurements. In each case, 10^{-5} M solutions of the BMI in benzene and in a 4×10^{-5} M solution of OA in 10 mM borate buffer were prepared and then excited at 349 nm. To eliminate the contribution from Raman scattering, the emission spectra of the solvents alone were recorded and subtracted from the emission spectra of the sample solutions. This correction was <5%.

Although excited-state decay rates can be obtained by single-photon counting, for such a large number of samples, we appealed to the fluorescence quantum yields Φ_f^X as a function of the substituent X (R_1 and R_2). Since $\Phi_f^X = k_f^X/k_{dt}^X$, where k_f^X is the rate constant for fluorescence, $k_{dt}^X = k_f^X + k_{nr}^X$ is the total rate constant for decay, and k_{nr}^X is the nonradiative decay rate constant, we obtain $\Phi_{rel}^X = \Phi_f^X/\Phi_f^H = (k_f^X/k_f^H)/(k_{dt}^X/k_{dt}^H)$, where Φ_f^H refers to the quantum yield for H/CH_3 (entry 1 in Table 1). Making the somewhat less than rigorous assumption that the oscillator strengths are not greatly affected by weakly perturbing substituents, we conclude that $k_f^X \approx k_f^H$, which gives $\Phi_{rel}^X \approx k_{dt}^H/k_{dt}^X$. Strictly speaking, we are interested in the ratio of nonradiative decay rates, k_{nr}^H/k_{nr}^X, but for relatively low Φ_f^X we can assume that total decay is dominated by internal conversion. The quantum yield was obtained by integrating the area under the emission curve from 360 to 650 nm.

Substituent effects are classically a function of electronic and steric effects, as represented by a linear free energy

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Table 1. Linear Free Energy and Fluorescence Parameters for Various BMIs Included in the OA Cavitand

		ΔV (Å^3)[a]				
R_1/R_2	σ	R_1	R_2	$10^{-3}\Phi_{OA}$[b]	$\log(\Phi^X_{rel})$[c]	fit[d]
H/Me	0.00	0.0	0.0	3.1	0.0	0.0
o-F/Me	0.29	4.6	0.0	69	1.3	0.6
m-F/Me	0.34	4.6	0.0	130	1.6	1.0
p-F/Me	0.06	4.6	0.0	2.2	−0.2	0.1
o-Cl/Me	0.50	13.8	0.0	78	1.4	1.1
m-Cl/Me	0.37	13.8	0.0	19	0.8	1.0
o-Br/Me	0.55	18.2	0.0	140	1.7	1.3
m-Br/Me	0.39	18.2	0.0	13	0.6	1.0
p-Br/Me	0.23	18.2	0.0	15	0.7	0.5
o-Me/Me	−0.13	18.3	0.0	1.2	−0.4	0.0
m-Me/Me	−0.07	18.3	0.0	1.0	−0.4	−0.4
p-Me/Me	−0.17	18.3	0.0	1.5	−0.3	−0.3
o-OMe/Me	−0.37	27.5	0.0	2.2	−0.2	−0.3
m-OMe/Me	0.12	27.5	0.0	3.7	0.1	0.1
p-OMe/Me	−0.27	27.5	0.0	0.93	−0.5	−0.5
o-CF_3/Me	0.81	32.0	0.0	440	2.2	2.0
m-CF_3/Me	0.43	32.0	0.0	75	1.4	1.1
H/Pr	0.00	0.00	37.0	15	0.7	0.4
o-Me/Et	−0.13	18.3	18.5	3.0	0.0	0.2
o-Me/Pr	−0.13	18.3	37.0	9.7	0.5	0.4
o-Me/Bu	−0.13	18.3	55.2	4.8	0.2	0.6
o-Me/Pn	−0.13	18.3	73.8	2.0	−0.2	0.8
m-Me/Pr	−0.07	18.3	37.0	1.8	−0.2	−0.2
p-Me/Pr	−0.17	18.3	37.0	2.1	−0.2	−0.2
m-F/Pr	0.34	4.6	37.0	220	1.8	1.2
p-F/Pr	0.06	4.6	37.0	7.2	0.4	0.2
o-Cl/Pr	0.50	13.8	37.0	160	1.7	1.5
m-Cl/Pr	0.37	13.8	37.0	20	0.8	1.2
o-Br/Pr	0.55	18.2	37.0	180	1.8	1.7
m-Br/Pr	0.39	18.2	37.0	7.5	0.4	1.2
p-Br/Pr	0.23	18.2	37.0	29	1.0	0.6
o-OMe/Pr	−0.37	27.5	37.0	8.8	0.5	0.1
m-OMe/Pr	0.12	27.5	37.0	11	0.5	0.3
p-OMe/Pr	−0.27	27.5	37.0	1.2	−0.4	−0.4
o-CF_3/Pr	0.81	32.0	37.0	150	1.7	2.4
m-CF_3/Pr	0.43	32.0	37.0	170	1.7	1.2

[a] Determined by DFT calculations. [b] Emission quantum yield in OA. [c] Relative emission quantum yield in OA with respect to H/Me. [d] Fit to $\log(\Phi^X_{rel})$ in eq 2 for OA.

relationship (LFER) (eq 1):

$$\log(\Phi^X_{rel}) \approx \log\left(\frac{k^H_{nr}}{k^X_{nr}}\right) = \sigma\cdot\rho + \alpha\cdot\chi \qquad (1)$$

Here σ is the Hammett substituent constant, ρ is the reaction constant, χ is a steric constant, and α is the related proportionality constant. In the case of two substituents, eq 1 can be modified as

$$\log(\Phi^X_{rel}) \approx \log\left(\frac{k^H_{nr}}{k^X_{nr}}\right) = \sigma\cdot\rho + \alpha\cdot\chi_1 + \beta\cdot\chi_2 \qquad (2)$$

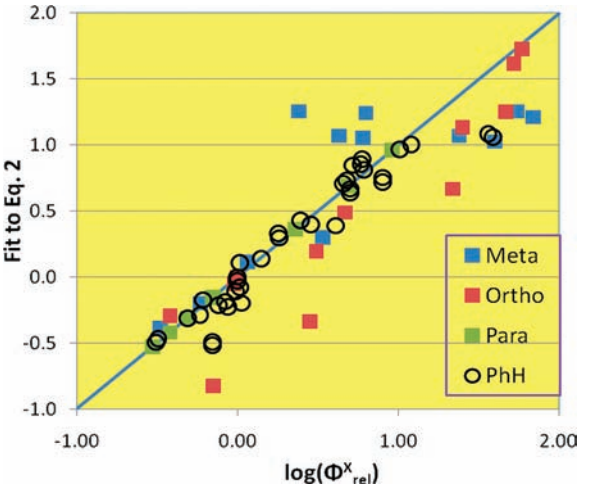

Figure 2. Fit of eq 2 to the experimental data.

where β is the proportionality constant for the steric constant of substituent 2. Steric constants have been represented in a variety of ways, including the Charton ν constants[8] and *A* values.[9] However, these generally capture only the steric hindrance of a substituent and not its total volume, which may be inappropriate for the effect within a confined space.[10] For instance, a methoxy group has a larger volume but a smaller *A* value than a methyl group because the nearest-neighbor atom is smaller. Instead, we deemed it more appropriate to represent χ_1 and χ_2 by the density functional theory (DFT)-calculated changes in molecular volume, ΔV, upon replacement of hydrogen with the substituent on benzene in the case of R_1 and the methyl group in toluene by the appropriate alkyl group in the case of R_2, respectively (Table 1). Thus ΔV for CH_3 in the case of R_1 is 18.3 Å^3 and in the case of R_2, 0.0 Å^3.

Use of the Hammett equation for excited-state processes has a somewhat checkered history, and a number of substituent constants have been proposed for photochemical processes.[11] As is generally the case for LFERs, we appeal to the success of the method at reproducing the data. Moreover, while LFERs are well-developed for meta and para substituents, the use of σ values for ortho substituents is fraught with problems, almost all of which have to do with dissecting electronic versus steric effects.[12] Thus, we adopted the ortho σ values developed by Tribble and Traynham,[13] which are based upon the apparently sterically independent chemical shifts of phenols.

Using benzene solvent as a proxy for the aromatic OA interior,[14] we performed a linear regression on eq 2 for each of the ortho, meta, and para substituents, obtaining the correlation (open circles) shown in Figure 2. In both benzene and OA, the CF_3 group did not correlate well and was neglected from the analysis. We now consider the interplay of the steric and electronic factors. Using the sums of the correlation factors, $\sum_i(\sigma_i\rho_i)^2$, $\sum_i(\alpha_i\chi_{1i})^2$, and $\sum_i(\beta_i\chi_{2i})^2$, we can estimate the percent contribution to the variance from each factor (see Table 2). First, we consider the substituent effects in benzene. We note that the electronic effect of the substituent, σ, dominates the decay, and the effects of the ortho and para substituents are similar, consistent with the usual ortho/para formalism. Second, the effects of the substituents are almost entirely electronic. The one exception, of course, is the ortho substituent, where 12% of the variance is due to the steric effect. Again, this is not surprising given the difficulty in dissecting steric from electronic effects in ortho substituents. Third, the

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Table 2. Relative Contributions of Electronic and Volumetric Factors

	ρ		α (R_1)		β (R_2)	
substituent	value	% contrib.	value	% contrib.	value	% contrib.
			in benzene			
ortho	1.3 ± 0.1	88	0.010 ± 0.003	12	-0.001 ± 0.002	<1%
meta	2.1 ± 0.2	99	0.001 ± 0.001	<1%	0.001 ± 0.002	<1%
para	1.8 ± 0.1	99	-0.001 ± 0.001	<1%	-0.001 ± 0.001	<1%
			in OA			
ortho	2.2 ± 0.3	82	0.018 ± 0.007	10	0.013 ± 0.005	8
meta	3.2 ± 0.7	87	-0.009 ± 0.011	12	0.005 ± 0.008	2
para	2.6 ± 0.3	93	0.005 ± 0.003	5	0.005 ± 0.004	2

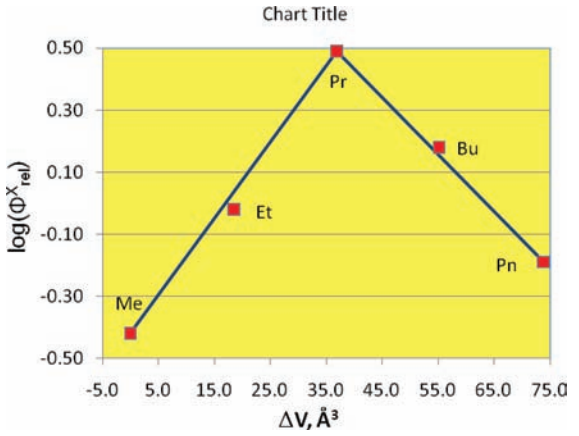

Figure 3. Effect of the *N*-alkyl substituent on $\log(\Phi^{X}_{rel})$.

N-alkyl substituent (R_2) has almost no effect, in agreement with little steric perturbation of the transition state. Finally, the ρ value is positive with magnitude >1, indicating substantial negative charge transfer to the aryl ring in the transition state for decay. Since the excited state (represented by a diradical in Figure 1) is known to have significant negative charge transfer to the imidazolidinone ring, it is not surprising that internal conversion would reflect that reverse charge transfer.

We now consider the effect of encapsulation. Unlike the results for benzene, the remote *N*-alkyl substituent has a significant effect (see Table 2), approaching that of the ortho substituent and decreasing in the order ortho > meta > para, as suggested by our previous results.[3] Curiously, as the volume increases in the series R_2 = Me, Et, Pr, Bu, and Pn (pentyl) while R_1 = Me, we see a linear increase and then a decrease in the fluorescence enhancement (see Figure 3), presumably reflecting the effect of locking and then overcrowding the capsule. Parenthetically, we note that the Charton ν constants and the *A* values for Pr, Bu, and Pn are nearly identical,[8] validating our use of molecular volume. For this reason, we restrict our consideration to Me and Pr as the *N*-alkyl groups but maintain the linearity with volume as the contribution to the LFE.

Comparing the steric effects for ortho, meta, and para, we see a decreasing steric effect for the phenyl substituent and a more dramatic decrease for the *N*-alkyl substituent. The "locking" effect we suggested earlier[3] is seen more clearly, with the ortho and meta substituents having a major steric effect and the para substituent, which cannot prevent aryl rotation, having less impact. Similarly, the ethyl group, at 36.8 Å^3, prevents encapsulation by OA, while the CF_3 group, at 32.0 Å^3, barely allows it when present in the ortho position.

Finally, we consider the nature of the Hammett ρ value in the capsule. With some variation for the ortho, meta, and para substituents, which presumably results from the use of a different set of σ values in each case, we note that $\rho(\mathrm{OA}) > \rho(\mathrm{PhH})$ in all cases. Thus, internal conversion within OA must require more charge transfer back to the ground state, resulting in the observed higher values for ρ within OA than in benzene solution. This may reflect a larger amount of twist within the capsule than in benzene. Thus, the role of the host molecule in rigidifying the chromophore is clearly demonstrated.

In other guest molecules, including biomolecules, we have observed rigidification of FP chromophores that is dependent upon structure in analogous ways.[15] We believe that such topological control of fluorescence represents a diverse method for creating molecular probes.

■ ASSOCIATED CONTENT

Supporting Information. Experimental information, synthetic details, characterization, and additional spectroscopic information. This material is available free of charge via the Internet at http://pubs.acs.org.

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