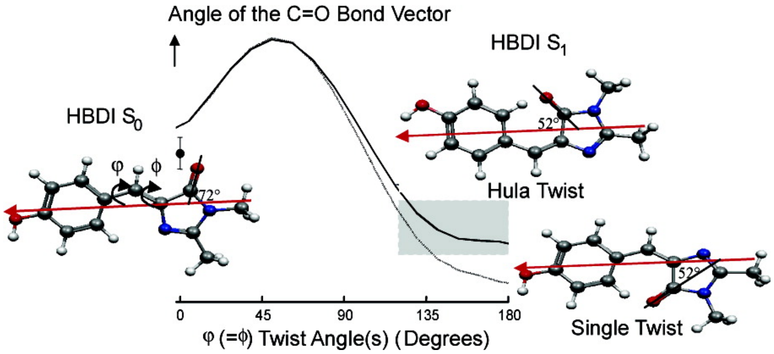
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

Excited-State Structure Determination of the Green Fluorescent Protein Chromophore

Anwar Usman, Omar F. Mohammed, Erik T. J. Nibbering, Jian Dong, Kyril M. Solntsev, and Laren M. Tolbert

J. Am. Chem. Soc., **2005**, 127 (32), 11214-11215• DOI: 10.1021/ja0520520 • Publication Date (Web): 23 July 2005



Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

JACS
COMMUNICATIONS

Excited-State Structure Determination of the Green Fluorescent Protein Chromophore

Anwar Usman,[†] Omar F. Mohammed,[†] Erik T. J. Nibbering,*[,†] Jian Dong,[§] Kyril M. Solntsev,[§] and Laren M. Tolbert[§]

Max Born Institut für Nichtlineare Optik und Kurzzeitspektroskopie, Max Born Strasse 2A,
D-12489 Berlin, Germany, and School of Chemistry and Biochemistry, Georgia Institute of Technology,
Atlanta, Georgia 30332-0400

Received March 31, 2005; E-mail: nibberin@mbi-berlin.de

In wild-type green fluorescent protein (GFP), an important photosensor protein used in bioimaging,[1,2] the photochemical dynamics of the chromophore incorporates a Förster cycle, where excitation of the photoacid chromophore is followed by proton transfer to the side groups of the protein pocket[3] through, apparently, an intramolecular charge transfer (ICT) state.[4] As a result, fluorescent emission with high efficiency (quantum yield $\Phi_{Fl} = 0.8$) occurs from the chromophore in its photobase form. In contrast, the chromophore of GFP, *p*-hydroxybenzylideneimidazolidinone (HBDI, see Figure 1), shows markedly different behavior, in which the ground state is recovered within picoseconds with high quantum yield ($\Phi_{IC} > 0.999$).[5] Twisting between the phenolate and imidazolidinone moieties of HBDI has been suggested as the underlying mechanism of this ultrafast internal conversion (IC) process.[6] Thus, understanding the microscopic mechanisms that underlie the dynamics and outcome of such photoinduced chemical reactions requires the determination of the molecular structure in the electronic excited state, which involves couplings with electronic ground and product states, thereby affecting the reaction quantum yield. The surrounding solvent may affect both the excited-state structure and the couplings with other states, often leading to pronounced solvent-induced changes in the reaction dynamics.

We now report on the ultrafast polarization-sensitive infrared (IR) spectroscopy[7] of the excited-state structure of HBDI, from which we can draw conclusions on the importance of twisting motions. Our focus is on the orientation of the transition moment of the IR-active carbonyl stretching vibration of HBDI relative to that of the electronic transition dipole moment, which indicates that the C═O vibration acts as a "spectator mode" for the relative orientation of the phenolate and imidazolidinone groups, from which we derive an effective near-complete twisting around the ethylenic bridge upon electronic excitation of HBDI. We include the anisotropy of the C═O vibration of HBDI in the S_0 and S_1 states for three charged configurations (anionic, neutral, and cationic form) in the fully deuterated solvents CD_3OD and dimethyl sulfoxide-d_6 (DMSO-d_6).

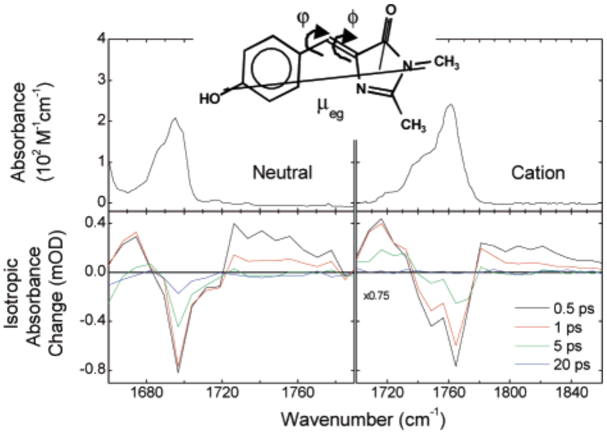


Figure 1. Steady-state and transient spectra showing the isotropic response of the C═O stretching bands in neutral and cationic HBDI in CD_3OD. Inset shows the HBDI chromophore with the direction of the electronic transition dipole moment, μ_{eg}, and the twisting angles, ϕ and φ.

In Figure 1, we show the transient difference absorbance response of HBDI in the spectral region of the C═O stretching vibration in CD_3OD (additional results can be found in the Supporting Information). The anisotropy free response, ΔA_{iso} (as defined by $\Delta A_{iso} = [\Delta A_{\|} + 2\Delta A_{\perp}]/3$), shows an absorbance decrease (bleach signals) corresponding to transitions of IR-active vibrations in the S_0 state, while an absorbance increase indicates vibrational bands of HBDI in the S_1 state at early pulse delays and red-shifted vibrational transitions in the S_0 state with high internal vibrational excess energy ("hot" ground state). The red-shifts are caused by anharmonic coupling between highly excited, presumably low-frequency, modes and the IR-active fingerprint vibrations probed in the experiment.[8,9] The decay of the C═O band in the S_1 state is equal to the population decay time. In contrast, the bleach decay associated with the C═O stretching vibration in the S_0 state is determined by both the $S_1 \rightarrow S_0$ IC rate and the subsequent cooling process when the excess internal vibrational energy is dissipated to the solvent. As a result, we observe a faster excited-state C═O stretching band decay than a ground-state C═O stretching bleach recovery. Figure 2 shows the decay S_0 state bleach and the S_1 state absorption signals of the C═O stretching mode for parallel and perpendicular polarizations for the neutral and cationic forms of HBDI in CD_3OD. These measurements provide the anisotropy, $D = \Delta A_{\|}/\Delta A_{\perp}$, from which the direction of the C═O stretching transition dipole moment can be derived. In Table S1, we summarize our findings for the C═O stretching mode.

For the S_0 state, we observe an increase in C═O stretching frequency going from anionic, via neutral, to cationic HBDI in CD_3OD and DMSO-d_6, in accordance with the literature,[10] reflecting an increase in C═O bond strength. Differences in electronic charge distributions are considered to be the cause of this,[10] but also, changes in hydrogen bonding interactions between the C═O and $-OH/-O^-$ groups with the solvent may play a role. Surprisingly, upon electronic excitation, the C═O stretching mode increases its frequency even further with 50 cm^{-1} for the neutral and cationic forms, which belies the presumed intramolecular charge-transfer nature of the excited state. We observe a rather broad band (width of 40−50 cm^{-1}) for the excited-state C═O stretching transition, hinting at significant inhomogeneous broadening. We were not able

---

[†] Max Born Institut für Nichtlineare Optik und Kurzzeitspektroskopie.
[§] Georgia Institute of Technology.

10.1021/ja0520520 CCC: $30.25

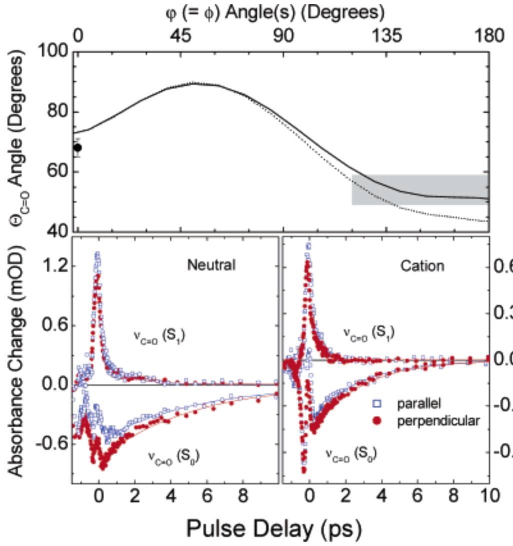


Figure 2. Transient polarization-sensitive response of the C═O stretching bands in the S_0 and S_1 states for the neutral and cationic species of HBDI in CD_3OD. Top panel shows the direction of the C═O bond vector, $\Theta_{C=O}$, with respect to the electronic transition dipole moment, μ_{eg}, as function of the twisting angle for single twist (dashed line) and hula twist (solid line). The observed value for $\Theta_{C=O}$ in ground-state HBDI, with $\varphi = 0$, is shown as a dot, whereas the gray bar indicates the possible configuration space for $\Theta_{C=O}$ in the excited state.

to identify for the anionic form of HBDI a distinct C═O stretching band in the S_1 state, which may imply much lower cross-sections, or more likely a frequency downshift by significant mixing with aromatic ring vibrations, resulting from mesomeric delocalization of the negative charge on the excited molecule.

Observation of the decay rates of the C═O stretching bands in both S_0 and S_1 states allows for an unequivocal determination of the IC and vibrational cooling rates in one experiment. Table S1 shows the results for HBDI in the different charged states. For neutral and anionic HBDI, we find an IC rate of $(1.2 \pm 0.2\ \mathrm{ps})^{-1}$. Even faster IC rates have been found for cationic HBDI $(0.6 \pm 0.2\ \mathrm{ps})^{-1}$. Similar values have been found with ultrafast fluorescence up-conversion [11] and electronic pump−dump−probe [12] spectroscopy, where, however, the signals are also affected by solvation dynamics. Conversely, the bleach recovery signals of the C═O stretching band are dominated by the slower vibrational cooling rate of about $(6\ \mathrm{ps})^{-1}$ to $(5\ \mathrm{ps})^{-1}$.

From our anisotropy measurements, we derive the angle between the electronic and vibrational transition dipole moments (see Table S1) to be around 70° in the S_0 state for all charged forms of HBDI. For neutral and cationic HBDI in the S_1 state, we find a lower value of around 52°. Comparison with the angle between the electronic transition dipole moment and the C═O bond vector shows perfect correspondence for the hypothesis that the C═O stretching vibration should be considered as a local mode without substantial mixing of other nuclear motional degrees of freedom, even though the chromophores have been calculated to be of planar geometries. On the basis of the fact that the C═O stretching exhibits a frequency up-shift upon electronic excitation, we feel confident that this hypothesis is even more valid in the S_1 state. Thus, using the explicit assumption of the C═O stretching transition moment coinciding with the C═O bond vector, we conclude that a significant reorientation of the C═O group occurs upon the $S_0 \rightarrow S_1$ excitation, and we further conclude that the ICT state involves little charge migration to the C═O bond.

A structural change in HBDI is likely to proceed by cis/trans isomerization by a single twist or a hula twist[13] of the ethylenic bond. We have considered the orientational changes of the C═O bond vector caused by single and hula twisting motions of the chromophore affecting the relative positions of the phenolate and imidazolidinone moieties (see Figure 2). We find that our estimated value for the C═O bond vector in the S_1 state can only be explained by a single twist of 120 ± 10° or a hula twist of 150 ± 30°. These values are much larger than the twisting angle of 90° found in quantum chemical calculations,[6,14] from which conical intersections at this twisting angle have been assigned as the efficient IC channel. Although, according to our findings, the equilibrium structure of HBDI in the S_1 state is even more displaced along the twisting coordinate upon electronic excitation than in the reported calculations, we conclude that this large displacement [8] makes the twisting coordinate a likely candidate for the efficient coupling between S_1 and S_0, leading to IC. The >90° twist also suggests the intervention of the *E*-isomer in the photochemistry of *Z*-HBDI, an isomerization reported by Tonge.[15] We note, however, that the rapid reisomerization to the *Z*-isomer even in the ground state suggests that the potential energy curve provides a strong restoring force. A highly twisted species together with the short excited-state lifetime suggests the involvement of an intersection in the deactivation of the S_1 state. Whether additional degrees of freedom play a decisive role in the IC process and other aspects of the photophysics[14,16,17] remains the subject of further studies.

Acknowledgment. This work has been supported by the DAAD, by a long term mission of the Egyptian Government, and by the NSF through Grant No. CHE-0096941.

Supporting Information Available: Experimental Section, additional results, and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509−544.
(2) Zimmer, M. *Chem. Rev.* **2002**, *102*, 759−781.
(3) Stoner-Ma, D.; Jaye, A. A.; Matousek, P.; Towrie, M.; Meech, S. R.; Tonge, P. J. *J. Am. Chem. Soc.* **2005**, *127*, 2864−2865.
(4) Grabowski, Z. R.; Rotkiewicz, K.; Rettig, W. *Chem. Rev.* **2003**, *103*, 3899−4031.
(5) Webber, N. M.; Litvinenko, K. L.; Meech, S. R. *J. Phys. Chem. B* **2001**, *105*, 8036−8039.
(6) Weber, W.; Helms, V.; McCammon, J. A.; Langhoff, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6177−6182.
(7) Lim, M.; Jackson, T. A.; Anfinrud, P. A. *Science* **1995**, *269*, 962−966.
(8) Fidder, H.; Rini, M.; Nibbering, E. T. J. *J. Am. Chem. Soc.* **2004**, *126*, 3789−3794.
(9) Nibbering, E. T. J.; Fidder, H.; Pines, E. *Annu. Rev. Phys. Chem.* **2005**, *56*, 337−367.
(10) He, X.; Bell, A. F.; Tonge, P. J. *J. Phys. Chem. B* **2002**, *106*, 6056−6066.
(11) Mandal, D.; Tahara, T.; Meech, S. R. *J. Phys. Chem. B* **2004**, *108*, 1102−1108.
(12) Vengris, M.; van Stokkum, I. H. M.; He, X.; Bell, A. F.; Tonge, P. J.; van Grondelle, R.; Larsen, D. S. *J. Phys. Chem. A* **2004**, *108*, 4587−4598.
(13) Liu, R. S. H.; Hammond, G. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11153−11158.
(14) Toniolo, A.; Olsen, S.; Manohar, L.; Martinez, T. J. *Faraday Discuss.* **2004**, *127*, 149−163.
(15) He, X.; Bell, A. F.; Tonge, P. J. *FEBS Lett.* **2003**, *549*, 35−38.
(16) Vendrell, O.; Gelabert, R.; Moreno, M.; Lluch, J. M. *Chem. Phys. Lett.* **2004**, *396*, 202−207.
(17) Altoe, P.; Bernardi, F.; Garavelli, M.; Orlandi, G.; Negri, F. *J. Am. Chem. Soc.* **2005**, *127*, 3952−3963.

JA0520520