Deriving molecular information from photoselection experiments of the green fluorescent protein using intense femtosecond pulses

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Introduction

Green fluorescent protein (GFP) from *A. victoria* is a widely used marker protein for a multitude of bioimaging techniques *in vivo* (Tsien, 1998)[14]. The photochemical characteristics of GFP arise from highly specific protein-chromophore interactions and are very different when the isolated *p*-hydroxybenzylidene-imidazolinone (HBDI) chromophore in the condensed state, where radiationless decay, associated with rapid twisting motions in the excited state (Usman *et al.*, 2005[15]; Weber *et al.*, 1999[20]), is dominant. In GFP the specific protein environment inhibits these and other rapid deactivation reactions (Usman *et al.*, 2005[15]; van Thor *et al.*, 2005[16]; van Thor, 2005[19]; Weber *et al.*, 1999[20]) and in addition provides specific H-bonding interactions with the HBDI chromophore that are functional in the light-driven reactions of GFP (figure 1).

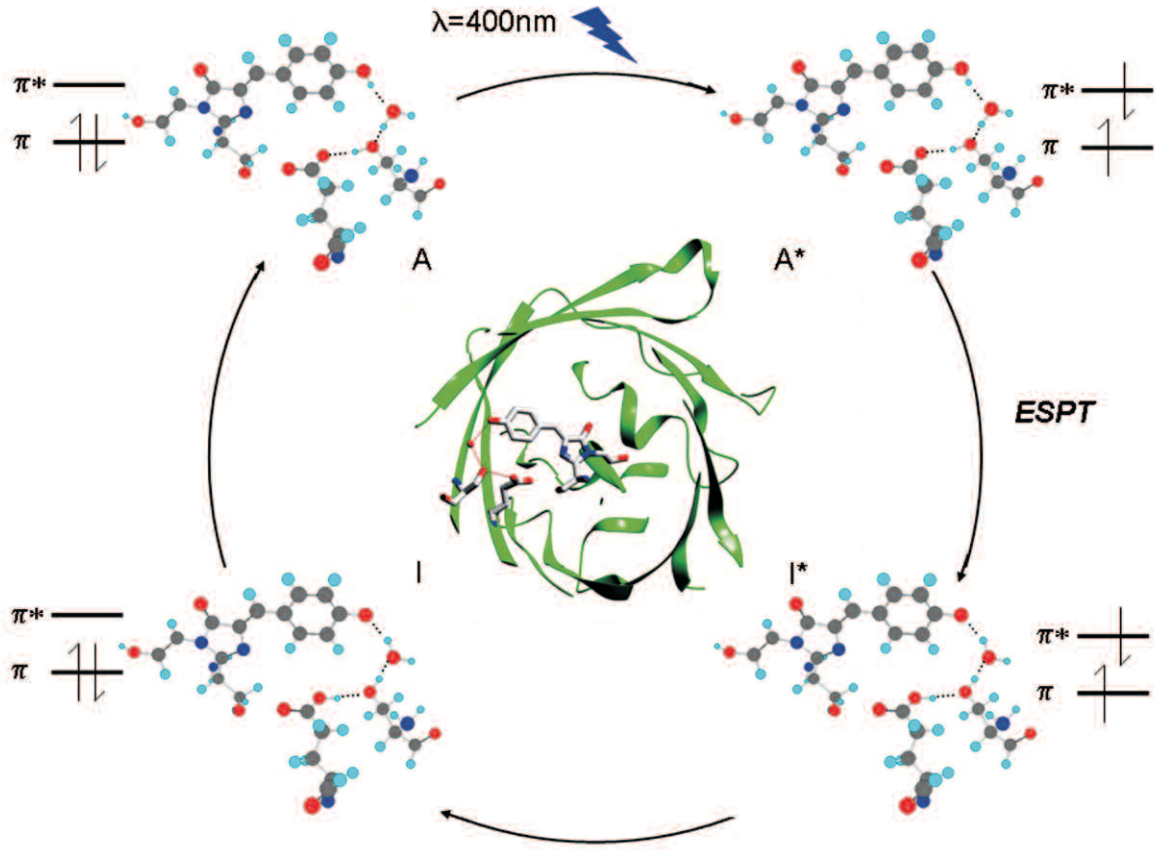

Figure 1. Overview of structural, H-bonding and electronic changes in the fluorescence photocycle. The phenolic oxygen of the HBDI chromophore is H-bonded indirectly to Glutamate 222 via a water molecule and a Serine 205. Ground state crystal structure coordinates 1W7S.pdb (van Thor *et al.*, 2005[16]) are shown. The electronically excited singlet state of the neutral and anion chromophore, A* and I* respectively, are indicated. Excited State Proton Transfer (ESPT) reactions occur during the transition between the A* and I* states, that have 10 and 75 ps time-constant in D_2O (Kennis *et al.*, 2004[6]; van Thor, 2005).[19]

It has been established that during the fluorescence photocycle, ps time-scale excited state proton transfer (ESPT) occurs through this network of H-bonding interactions (Chattoraj *et al.*, 1996[2]; Stoner-Ma, 2005[12]; Stoner-Ma *et al.*, 2006[13]; van Thor *et al.*, 2005[16]; van Thor, 2005[19]). Ultrafast mid-IR spectroscopy experiments have identified the buried carboxylate of E222 as the transient proton acceptor, from the analysis of H/D exchange effects and site specific mutation E222D (Stoner-Ma, 2005[12]; Stoner-Ma *et al.*, 2006[13]; van Thor *et al.*, 2005[16]; van Thor, 2005[19]). Additionally, the electrostatic rearrangements associated with the rapid proton transfer reaction have been shown to trigger a protein structural response on picosecond time-scale, and particularly of Q69 in the chromophore binding site (van Thor *et al.*, 2005[16]; van Thor *et al.*, 2008[17]; van Thor and Sage, 2006).[18]

We report on photoselection measurements that are extended from those previously reported on selected ground state bands in GFP (Stoner-Ma *et al.*, 2006)[13] and HBDI (Usman *et al.*, 2005[15]) to include the photocycle intermediates, we apply significant corrections to the results for the finite bleach that occurs with fs pulsed visible excitation and we correct reported theoretical calculations of vibrational transition dipole vectors (Stoner-Ma *et al.*, 2006[13]) for the interpretation of vibrational anisotropy, in addition to extending those calculations to intermediates and excited states. Necessary corrections to infrared anisotropy measurements, as has been demonstrated previously to significantly affect also molecular interpretation of CO binding in myoglobin (Lim *et al.*, 1995[8]), may be dominated by the finite bleach effect but can have a contribution from depth averaging of the second moment in absorbing samples (Lim, 2002[7]; Lim *et al.*, 1995)[8]. Molecular interpretation is additionally affected, even for relatively localised vibrations such as the C-O stretching mode of heme bound carbon monoxide in myoglobin, by mode mixing which can give rise to deviations between the transition dipole moment vector and the corresponding bond vector (Spiro and Kozlovski, 1998[11]), or from a nonlinear dipole response in the normal mode (Spiro and Kozlovski, 1998).[11]

Results and discussion

We performed time-resolved and polarisation-resolved mid-infrared spectroscopy measurements of the photocycle transitions of the wild-type GFP with fs excitation of the neutral ground state A at 400 nm. Using a single sample that was flowed in a closed circulating system, TRIR data were collected with high S/N ratio at horizontal, vertical and magic angle orientations relative to the polarised probe beam, in 6 overlapping spectral windows between 1775 and 1250 cm^{-1}. The resulting data were globally fitted with a photocycle model essentially as previously reported (van Thor, 2005)[19]. In D_2O, this leads to parallel excited state deuteron transfer reactions with 10 and 75 ps time constants, forming the deprotonated radiative intermediate I* that has a 3 ns lifetime. After decay of the I* state to the I_2 electronic ground state, reprotonation to reform the neutral ground state A has a large H/D kinetic isotope effect of ~10 and occurs with a time constant of 5 ns. This leads to transient accumulation of the I_2 state in the photocycle which is not observed in H_2O (Kennis *et al.*, 2004[6]; van Thor, 2005)[19]. Global fitting thus represents the kinetic data in a few polarisation-sensitive species associated difference spectra, that are the basis for molecular interpretation.

The quantitative interpretation of photoselection measurements (Ansari and Szabo, 1993[1]; Lim, 2002[7]; Lim *et al.*, 1995[8]; van Thor *et al.*, 2008[17]) relies on the expression

$$\left\langle P_2(\hat{\mu}_2 \cdot \hat{e}_1) \right\rangle = \left\langle P_2(\hat{\mu}_2 \cdot \hat{\mu}_1) \right\rangle \left\langle P_2(\hat{\mu}_1 \cdot \hat{e}_1) \right\rangle \quad (1)$$

which follows from the spherical harmonic addition theorem. Here, $P_2(x) = \frac{1}{2}(3x^2 - 1)$ is the second order Legendre polynomial and $\langle \cdots \rangle$ indicates an ensemble average. Eq. 1 quantitatively relates polariszed absorption measurements of a vibrational transition with transition dipole parallel toΔ, to the angular displacement of $\hat{\mu}_2$ from a molecular reference axis $\hat{\mu}_1$, which we can choose to be the optical transition dipole vector. The factor $\langle P_2(\hat{\mu}_2 \cdot \hat{\mu}_1) \rangle$ contains the desired molecular information and in simple cases, $\hat{\mu}_2 \cdot \hat{\mu}_1$ is identical for all molecules. $\langle P_2(\hat{\mu}_2 \cdot \hat{e}_1) \rangle$ is equal to the measured anisotropy $r = (\Delta A_{||} - \Delta A_{\perp})/(\Delta A_{||} + 2\Delta A_{\perp})$ determined from the absorption changes $\Delta A_{||} = 3\Delta A(\hat{\mu}_2 \cdot \hat{e}_1)^2$ and $\Delta A_{\perp} = 3\Delta A[1-(\hat{\mu}_2 \cdot \hat{e}_1)^2]/2$ with polarisations parallel or perpendicular, respectively, to the laboratory direction $\hat{e}_1$.

The primary obstacle in photoselection experiments, where the direction $\hat{e}_1$ is the polarisation of the light that is used to prepare the oriented ensemble, is determination of the optical anisotropy $\langle P_2(\hat{\mu}_1 \cdot \hat{e}_1) \rangle$ which measures the lowest non-zero moment of the orientation distribution of $\hat{\mu}_1$ with respect to $\hat{e}_1$. In the absence of a direct and reliable measurement, $\langle P2(\hat{\mu}_1 \cdot \hat{e}_1) \rangle$ may be calculated if the power density used and the optical cross sections are accurately known. For a light-driven unimolecular reaction $A \xrightarrow{k} B$ that occurs with quantum yield ϕ, the rate $k(t) = 3J(t)\sigma\phi(\hat{\mu}_1 \cdot \hat{e}_1)^2$ depends on the orientation of the molecular transition moment $\hat{\mu}_1$ relative to the polarisation $\hat{e}_1$. In this instance the photon flux density J (t) is significant over a period much shorter (150 fs) than the rotational correlation time (15 ns), so that $\hat{\mu}_1$ is time-independent. The fraction $n(x) = [1 - \exp(-\int k(t, x)dt)] = 1 - \exp(N_0 x^2)$ of molecules excited is then expressed in terms of a factor $N_0 = 3\bar{J}\sigma\phi$ proportional to the total photon density $\bar{J} = \int J\,dt$ and the cosine, $x = \hat{\mu}_1 \cdot \hat{e}_1$, of the angle between $\hat{\mu}_1$ and $\hat{e}_1$. This model provides an estimate of the second moment

$$\left\langle P_2(\hat{\mu}_1 \cdot \hat{e}_1) \right\rangle = \frac{\frac{1}{2}\int_{-1}^{1} dx\, n(x) P_2(x)}{\frac{1}{2}\int_{-1}^{1} dx\, n(x)} \quad (2)$$

of the orientational distribution appearing in Eq. 1. If the sample absorbance A is significant, the photolysed fraction and optical anisotropy vary as a function of depth z in the sample because of the attenuation of the intensity $\bar{J}(z) = \bar{J}_0 10^{-Az/d}$. In this case, it is necessary to average over the sample depth as well. Lim (Lim, 2002[7]; Lim *et al.*, 1995[8]; Lim *et al.*, 2004[9]) has shown that the depth-averaged second moment

$$\overline{\langle P_2(\hat{\mu}_1 \cdot \hat{e}_1) \rangle} = \int_0^d dz\, n_0(z) \langle P_2(\hat{\mu}_1 \cdot \hat{e}_1) \rangle \qquad (3)$$

must be weighted by the fractional photolysis n_0 (z) in each layer, assuming that the cross sections of molecules A and B are the same at the excitation wavelength. This correction was neglected because time resolved absorption spectroscopy showed a significant ground state bleach at 400 fs delay after 400 nm excitation (Kennis *et al.*, 2004)[6]. In addition, with a ground state absorption OD_{400} = 0.176, the photolysis levels used in this study would lead to corrections in the order of about 1% for equal excited state cross section, which is considered less then the measurement uncertainty.

For molecular interpretation of the photoselection measurements in addition to corrections of the infrared absorption dichroism, also the optical transition dipole vector $\hat{\mu}_1$ must be known. A reported optical crystallography study (Shi *et al.*, 2007[10]) that used $P2_12_12_1$ crystals, provides two solutions at 1.5° and 63.4° (assuming measurement along {100}) and two at 76.0° and -21.3° (assuming measurement along {010}) relative to a molecular reference axis which is in the chromophore plane and connects the OH and O_2 chromophore atoms of which the solution at 1.5° is closest to the calculation at the TD-DFT B3LYP/6-311+G(d,p) level. In our analysis we find the best correspondence with the TD-DFT calculation. Vibrational mode assignments particularly of the chromophore are based on harmonic frequency calculations at the DFT level, in combination with isotopic substitutions in model compounds (Esposito, 2001[3]; He *et al.*, 2002[5]) for neutral and anionic ground state, and were addressed recently for the corresponding excited states using single excitation Configuration Interaction (CIS) (van Thor *et al.*, 2008[17]). A comparison of the experimental dichroism and the corresponding mode assignment with theoretical calculation, used calculation of the dipole gradients with the Gaussian 03 software package (Frisch *et al.*, 2004[4]). Infrared transition dipoles for selected normal modes when plotted in GaussView 3.0 were found to be in error. In addition, these resulting dipole vectors also did not correspond to selected examples of similar calculations done with the Gaussian 98 package previously reported (Stoner-Ma *et al.*, 2006[13]). Therefore, infrared transition dipole vectors were calculated correctly using a finite difference calculation from the molecular dipoles with normal mode displacements <0.1 KT either side of the equilibrium geometry (van Thor *et al.*, 2008[17]). Further investigation implicated GaussView as the source of the error, as correct analytical dipole derivatives are printed using the iop(7/32=3,7/33=1) option in Gaussian 03 (Figure 5). The best agreement between experimental and theoretical dichroism was found for the high frequency chromophore modes such as the A state C=C mode, the phenol 1 modes and the A* and I* phenol 3 modes (van Thor *et al.*, 2008[17]). There are also cases where theoretical values do not match experiment for proposed assignments. A number of those can be explained by the inaccuracy of the experimental determination, however this can not explain

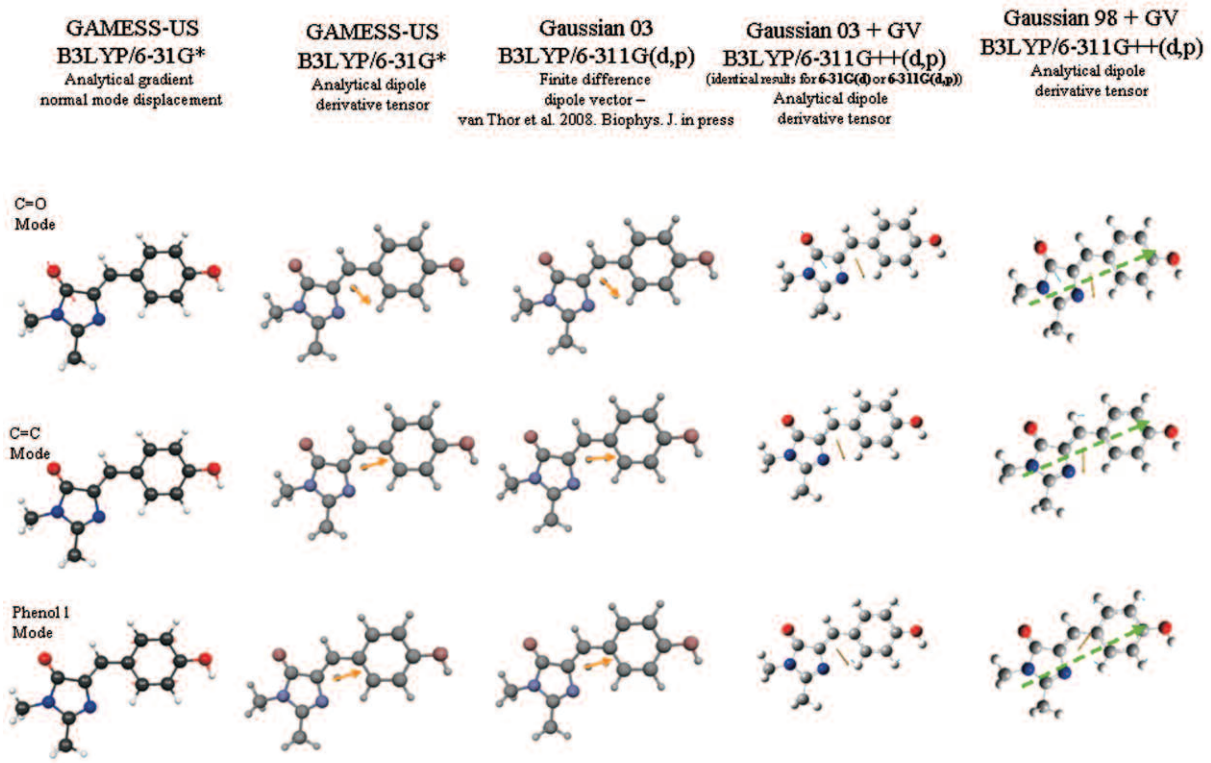

Figure 2. Comparison of incorrect analytical vibrational transition dipole vectors plotted by GaussView for C=O, C=C and Phenol 1 modes of the HBDI chromophore of GFP. A finite difference calculation using Gaussian provided the correct dipole vectors.

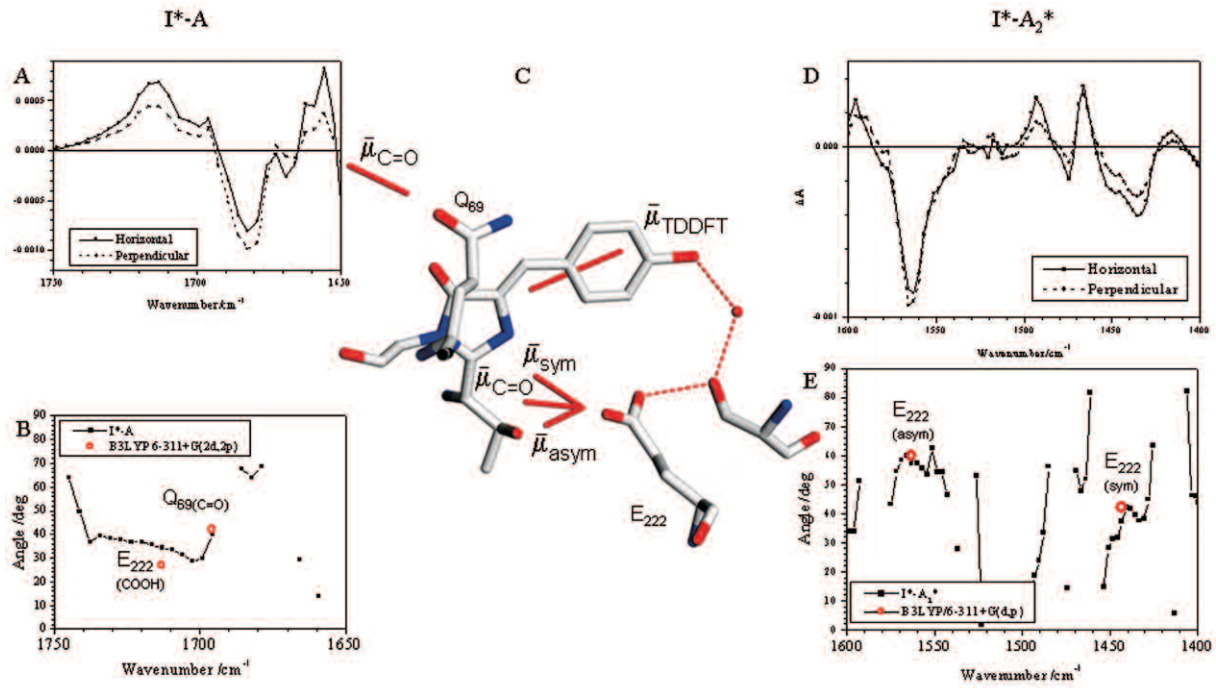

Figure 3. Correspondence of theoretical transition dipole moments of assigned amino acid normal modes with polarisation dependent species associated difference spectra.

a) The 1750-1650 cm^{-1} region of the, finite bleach corrected, polarisation dependent I* minus A difference spectrum of the wild type. COOH and C=O stretching vibrations of E222 and Q69 are assigned at 1711 and 1695 cm^{-1} respectively.

b) Calculated and experimental angles θ in the 1750-1650 cm^{-1} region of the polarisation dependent I* minus A difference spectrum.

c) Structural arrangement determined in the ground state with coordinates taken from 1W7S (van Thor, 2005[19]). The optical transition dipole moment $\hat{\mu}_1$ obtained from a TD-DFT B3LYP/6-311+G(d,p) calculation is shown an in-plane vector. The vibrational transition dipole moment vectors $\hat{\mu}_2$ from finite difference calculations at the B3LYP/6-311+G(d,p) are placed in the X-ray geometry.

d) The 1600-1400 cm^{-1} region of the, finite bleach corrected, polarisation dependent I* minus A2* difference spectrum of the wild type.

e) Theoretical and experimental angles θ in the 1600-1400 cm^{-1} region of the polarisation dependent I* minus A2* difference spectrum.

the differences in all cases such as the phenol 2 mode in the A states for which the theoretical value is 71° (or 55 ° using the experimental $\hat{\mu}_1$) and experimentally a value of 22° is determined, in spite of an intense and isolated difference band (van Thor *et al.*, 2008).[17]

Assignment of 1711, 1695, 1570 and 1450 cm^{-1} bands to amino acid E222 (COOH), Q69, E222 ν_{sym} and E222 ν_{asym} modes respectively have been established (Stoner-Ma, 2005[12]; van Thor *et al.*, 2005[16]; van Thor *et al.*, 2008[17]; van Thor, 2005[18]). Good correspondence is found between experimental measurements that are carefully corrected for the finite bleach and empirically for excitation intensity, with theoretical values obtained at the B3LYP/6-311+G(d,p) level by the finite difference method placed in the X-ray geometry (figure 6). We note that, with the exception of the E222 (COOH) band, overlap between the chromophore and amino acid bands is likely to lead to further uncertainty in the experimental determination of values for the angles θ between $\hat{\mu}_2$ and $\hat{\mu}_1$. Nevertheless there appears to be good correspondence, particularly for the ν_{sym} and ν_{asym} modes of E222, presumably because these bands locally dominate the species dependent difference spectrum (Figure 6D). The photoselection experiments therefore lead to a type of ultrafast structure solution of individual amino acids in the active site of GFP.

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