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Resonance CARS Study of the Structure of "Green" and "Red" Chromophores within the Red Fluorescent Protein DsRed

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Green fluorescent protein (GFP) from the jellyfish *Aequorea*, together with its mutants, has become a widely used fluorescent probe for molecular and cellular biology studies.[1] Recent cloning of the red fluorescent protein DsRed, originally called drFP583, from the *Discosoma* genus of reef coral[2] has significantly enhanced the palette of fluorescent proteins. DsRed, having red-shifted emission maximum, has attracted intense interest[3−10] as a potential expression marker and convenient fusion partner for GFP mutants for double-labeling experiments. DsRed possesses many features advantageous for biological applications such as high brightness, resistance to photobleaching, and insensitivity to pH changes.[4] However, two major deficiencies have also been revealed, that is, strong oligomerization and slow and incomplete maturation of the red fluorescence.[3−10] Even after prolonged maturation, DsRed contains a substantial amount of the green chromophore; while the 559-nm absorption and 583-nm emission maxima of DsRed belong to the mature "red" species, the absorption shoulder around 487 nm and weak emission peak at 500 nm are attributed to the immature "green" species.

We have probed the structure of DsRed by polarization-sensitive multiplex resonance coherent anti-Stokes Raman scattering (CARS) spectroscopy,[11−13] a powerful tool for studying the vibrational spectra of fluorescent molecules.[14,15] Although Raman spectra of wild-type GFP, its mutants, and a model GFP chromophore have already been reported,[16−18] no vibrational data on DsRed have been published yet. Moreover, because of a strong fluorescence background, CARS spectroscopy is the only choice for selective probing of different DsRed species in resonance with their visible absorption bands. Besides DsRed, a well-known GFP mutant, S65T-GFP, has also been studied as a reference.

The CARS spectrometer has been described elsewhere.[12,15] Briefly, the second harmonics of a mode-locked Nd:YLF laser was used for synchronous pumping of two cavity-dumped dye lasers (2 MHz, ∼15 ps, Rh560, Rh590 and/or DCM-filled). The pump dye laser (ω_1) was operating in a narrowband mode, while the Stokes laser (ω_2) was in a broadband mode. The CARS signal at $\omega_{as} = 2\omega_1 - \omega_2$ was dispersed by a polychromator with ∼4 cm^{-1} spectral resolution and registered by a CCD. Polarization of the Stokes beam ($\mathbf{e}_2$) was adjusted in a vertical plane, making an angle of 60° with the pump beam ($\mathbf{e}_1$). The plane of analyzer transmission ($\mathbf{e}_{as}$) was set at an angle of 65° to $\mathbf{e}_1$. Laser beams were focused by a 40× objective (NA = 0.4) into a 200-μm quartz capillary filled with protein. A flowing system with a peristaltic pump was used for constant protein replacement. The overall accumulation time for one spectrum was 1 min. Band frequencies were determined with ±2 cm^{-1} accuracy.

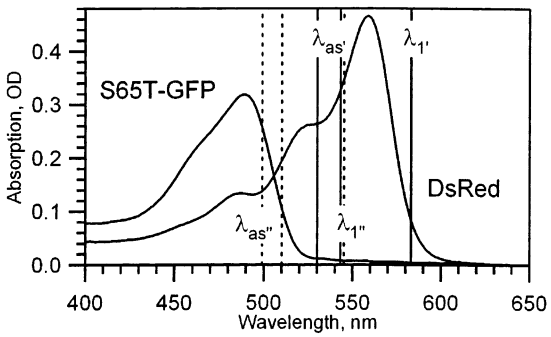

Figure 1. Position of excitation wavelengths on the absorption spectra of investigated proteins in two different CARS experiments.

Recombinant DsRed and S65T-GFP proteins with a 6-histidine tag were expressed in *Escherichia coli* and purified on a Ni-chelating resin (Ni−NTA-Agarose, Qiagen, Hilden, Germany) using standard procedures. Proteins were dissolved in 10 mM Tris buffer (pH8, 0.1 M NaCl) to concentrations of 8×10^{-5} M for DsRed and 7×10^{-5} M for S65T-GFP.

For selective probing of the "red" DsRed form, excitation at $\lambda_{1'} = 583$ nm (30 nJ/pulse) was chosen so that the anti-Stokes signal covered the $\lambda_{as'} = 530-544$ nm range (Figure 1). For probing the "green" DsRed form and S65T-GFP, excitation at $\lambda_{1''} = 545$ nm (5 nJ/pulse) was used, with $\lambda_{as''} = 499-510$ nm. It should be noted that in CARS, an electronic enhancement occurs in resonance with both λ_1 and λ_{as}.

The red-shifted absorption maximum of the mature DsRed species (559 nm) was explained[5,7] by an extension of the GFP-like π-bonding chromophore system by two electron-withdrawing double bonds after addition of the −C═N−C═O group at the 2-position of the imidazolidinone, which occurs during isomerization around a cis peptide bond between Phe 65 and Gln 66. At the same time, the absorption maximum of the immature DsRed species (487 nm) nearly coincides with that of S65T-GFP (489 nm). Thus, the "green" form of DsRed is usually referred to as a "GFP-like chromophore"[5,7] although such a statement has not been proven by direct structural data.

Figure 2 presents experimental results. Recorded CARS spectra have been normalized to the nonresonant background signal from water and then fitted with a third-order nonlinear susceptibility model function assuming complex Lorentzian line shapes.[13] The CARS spectrum of S65T-GFP (Figure 2A) has been fitted using

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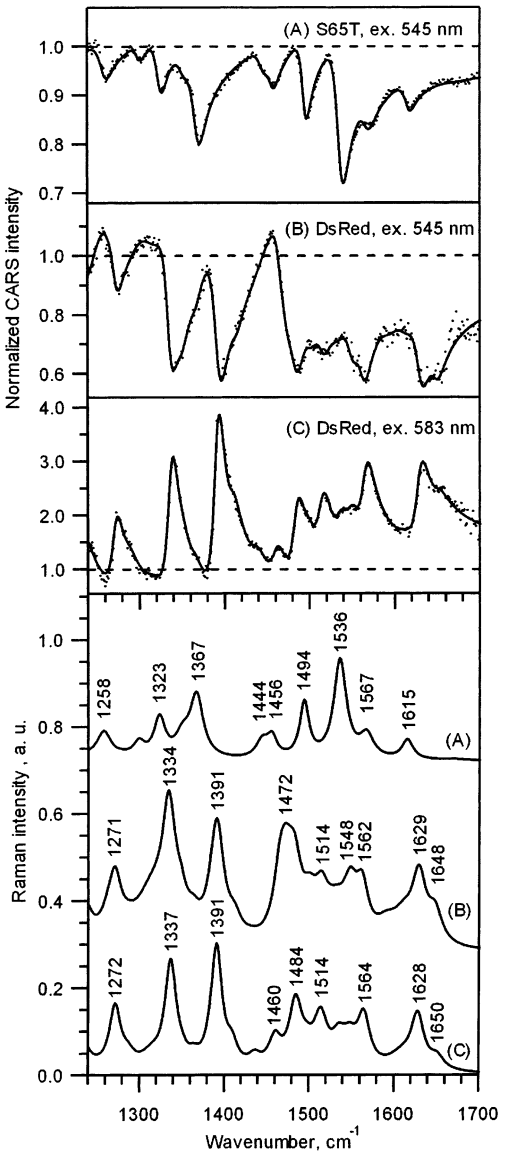

Figure 2. CARS (upper panel) and corresponding Raman (lower panel) spectra of S65T-GFP with excitation at $\lambda_1 = 545$ nm (A), DsRed with excitation at $\lambda_1 = 545$ nm (B), and $\lambda_1 = 583$ nm (C). Dots represent normalized experimental points, solid curves were derived from the fit.

14 bands and an electronic phase factor $\theta = 40°$. The CARS spectra of DsRed have been fitted using 22 bands and phase factors $\theta = 34°$ (Figure 2B) and $\theta = -160°$ (Figure 2C). Raman spectra have then been reconstructed from the CARS spectra using the best-fit parameters, and the frequencies of the prominent bands are indicated in the lower panel of Figure 2.

First, for S65T-GFP, we draw attention to the remarkable resemblance of the Raman spectrum obtained with 545-nm excitation (Figure 2A) to the published Raman spectrum recorded with 752-nm excitation (Figure 7C of ref 16). In fact, all the bands observed in the resonance Raman spectrum are also observed at preresonant conditions, that is, independent of the excitation wavelength, as is expected[17] considering the low symmetry of the GFP chromophore and taking into account that S65T-GFP exists exclusively in an anionic form at pH 8.[1]

Second, the vibrational spectra of S65T-GFP (Figure 2A) and "red" DsRed (Figure 2C) chromophores are clearly different. Although certain similarities can be found, in particular in the 1240−1420 cm^{-1} range, generally the DsRed spectrum exhibits more features. Moreover, the strongest band in the S65T-GFP spectrum at 1536 cm^{-1}, which is the dominant feature for all GFP chromophores,[16−19] cannot be easily identified in the DsRed spectrum. This finding can be rationalized by the fact that the "red" DsRed chromophore possesses an extended structure; since all the Raman modes in the region 1400−1600 cm^{-1} are coupled, delocalized C−C, C−N, and C−O stretch vibrations,[19] the chromophore extension must lead to a different intensity distribution between these modes.

Finally, for DsRed, in contrast to S65T-GFP, a shift of the excitation wavelength (from 583 nm, Figure 2C to 545 nm, Figure 2B) causes noticeable changes in the overall spectral contour. Most prominent is the doubling of the bands in 1450−1600 cm^{-1} region and a broadening of the contour around 1334 cm^{-1}. We propose that both "red" and "green" DsRed species contribute to the vibrational spectrum at any excitation wavelength, with the contribution from the "green" species being enhanced for 545-nm excitation. Assuming a GFP-like structure, one would expect the immature "green" DsRed species to have a vibrational spectrum similar to that of S65T-GFP. However, this is not the case; the vibrational pattern of the "green" form of DsRed resembles that of the "red" form, albeit with relatively small frequency shifts for a number of bands. Therefore, our CARS data reveal that both "green" and "red" DsRed species possess an extended chromophore structure, which differs from that of the GFP chromophore. Consequently, our data suggest that the π-bonding chromophore system extension during isomerization around the cis peptide bond between Phe 65 and Gln 66 is a necessary but not sufficient step in DsRed chromophore maturation.

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