Circular dichroism spectroscopy of fluorescent proteins

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Abstract Circular dichroism (CD) spectra have been obtained from several variants of green fluorescent protein: blue fluorescent protein (BFP), enhanced cyan fluorescent protein (CFP), enhanced green fluorescent protein (GFP), enhanced yellow fluorescent protein (YFP), all from *Aequorea victoria*, and the red fluorescent protein from the coral species *Discosoma* (DsRed). We demonstrate that CD spectra in the spectral fingerprint region of the chromophore yield spectra that after normalization are not coincident with the normalized absorbance spectra of GFP, YFP and DsRed. On the other hand, the CD spectra of BFP and CFP coincide with the absorbance spectra. The resolution of absorption and CD spectra into Gaussian bands confirmed the location of the different electronic band positions of GFP and YFP as reported in the literature using other techniques. In the case of BFP and CFP the location of Gaussian bands provided information of the vibrational progression of the electronic absorption bands. The CD spectrum of DsRed is anomalous in the sense that the major CD band has a clear excitonic character. Far-UV CD spectra of GFP confirmed the presence of the high β-sheet content of the polypeptide chain in the three-dimensional structure.

Key words: Circulair dichroism; Green fluorescent protein; Rotational strength; Excitation splitting; Secondary structure; Transition energies

1. Introduction

The green fluorescent protein (GFP) together with its differently colored mutants has spurred an exponentially increased utilization in cell biology as a natural, brightly fluorescent marker for gene expression, localization of gene products, intracellular pH and diffusion measurements and intracellular protein–protein interaction via fluorescence resonance energy transfer [1–14]. In order to understand the complex photophysics of GFP and its mutants, a considerable number of optical spectroscopic studies have been published, covering sub-picosecond time-resolved fluorescence [15–17], picosecond time-resolved fluorescence [18–20], spectral hole-burning at cryogenic temperature [21,22], fluorescence correlation spectroscopy [23–27] and single-molecule fluorescence spectroscopy [28–32]. The majority of these studies showed complex photophysics of GFP, which could be accounted for in part by the available three-dimensional structures [33–36]. Based on femtosecond fluorescence up-conversion experiments on wild-type GFP, Chattoraj et al. [15] proposed for the first time a photophysical scheme of three states: one stable protonated state A (absorbing at 395 nm), one stable deprotonated state B (absorbing at 475 nm) and an intermediate state I, which is supposed to be an unrelaxed form of B. When the ground state A (absorbing at 395 nm) is excited, very fast excited-state proton transfer (4 ps) takes place from A* to an intermediate, deprotonated state I*. The I* state returns to the ground state I via fluorescence (around 510 nm) or, occasionally, relaxes to the B* state followed by fluorescence (also around 510 nm). The latter mechanism was confirmed by both femtosecond and picosecond time-resolved spectroscopic experiments carried out by Lossau et al. [16] for wild-type and enhanced GFPs. The three forms, together with their 0-0 transition energies, were identified by spectral hole-burning experiments at cryogenic temperature [21,22]. The fluorescence lifetime, attributed to the I form, is slightly longer than the one attributed to the B form (3.3 and 2.8 ns, respectively) [18,20]. Quantum chemical calculations on cis-trans photoisomerization of the chromophore indicated the presence of a zwitterionic form of GFP, in addition to protonated and anionic forms, playing a role in internal conversion processes [37,38].

The present study is directed towards an investigation of the chirality of the chromophore induced by the protein matrix which can be investigated with circular dichroism (CD) spectroscopy of enhanced GFP from *Aequorea victoria* and the following (enhanced) fluorescent protein (FP) mutants derived from it: blue fluorescent protein (BFP), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) and the red fluorescent protein from the coral species *Discosoma* (DsRed) [39]. The investigation comprises the acquisition of both CD and absorbance spectra in the fingerprint spectral region of the chromophore. In view of the fact that the spectroscopic properties of the chromophoric group in the FPs are still not completely understood, such a study is of interest as the intracellular fluorescence applications of FPs must be correctly interpreted. CD spectra exhibit additional features that are not present or hidden in the corresponding light absorption spectra [40,41]. It would then be possible to assign the location of the different electronic bands and compare the band

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positions with the ones obtained with other techniques [21,22]. In the case of DsRed, this protein shows a tendency to oligomerize to tetramers even at low concentration [42–44]. In this particular case, CD spectra would reveal the possible electronic coupling between the chromophoric groups via the presence of an exciton band. Recently, much research has been carried out on the photochemical, photophysical, spectroscopic and other properties of DsRed because of its favorable use as a brightly red fluorescent marker in cell biological applications [45–50]. The crystal structures of DsRed have been recently reported and they confirmed the tetrameric structure [51,52]. Finally, we demonstrate that GFP is a potential secondary-structure protein standard for high β-sheet content when far-UV CD spectra are used to analyze the secondary structural content of a protein.

2. Materials and methods

The cDNA of GFP was kindly provided by Arjen Schots, Laboratory of Molecular Recognition and Antibody Technology, Wageningen University, and expression and isolation of GFP was performed as described in [53]. BFP was a generous gift from Petra Völler and Holger Lill of the Department of Structural Biology, Vrije Universiteit Amsterdam. The open reading frame of CFP and YFP was amplified by polymerase chain reaction (PCR) from the full-length cDNAs and was cloned into the pTYB11 vector (New England Biolabs, Impact vector system). The purification of the two FPs was performed as described in the manual of New England Biolabs, Impact vector system. DsRed was obtained from Clontech and via PCR a His tag was introduced. Isolation of protein was performed according to the standard Ni-NTA procedures.

CD experiments were performed on a JASCO J-715 spectropolarimeter with a Jasco PTC 348 WI temperature controller. The visible-near-UV spectra (600–300 nm) were obtained in 1-cm path length quartz cuvettes with black side walls filled with 0.5 ml relatively concentrated FP solution in 0.1 M Tris–HCl buffer containing 100 mM KCl at pH 8.0. Four to nine spectra with a resolution of 1 nm, a scan speed of 50 nm/min and a response time of 1 s were averaged. Buffer blank spectra, obtained at identical conditions, were subtracted. The instrument also allowed the absorption spectra to be simultaneously collected, which are presented together with the CD spectra of the FP chromophore. The resolution of absorbance and CD spectra in a minimal number of Gaussian bands has been performed by IGOR-Pro (WaveMetrics) software. The far-UV spectra (240–190 nm) were obtained in quartz cuvettes of 1-mm path length and filled with 0.3 ml 2.4 μM GFP in 0.01 M sodium phosphate buffer at pH 7.5. Nine spectra with a resolution of 1 nm, a scan speed of 50 nm/min and a response time of 1 s were accumulated and averaged. Buffer blank spectra, obtained at identical conditions, were subtracted. The secondary structure content of GFP from the far-UV CD spectra was determined via ridge-regression analysis with the CONTIN program based on a set of 16 protein structures [40,54,55].

The temperature of all experiments was 293 K. All samples were freshly prepared prior to the measurements.

3. Results and discussion

3.1. CD spectra

In Fig. 1 the CD spectra of the FPs in the near-UV and visible spectral range are presented together with the simultaneously measured absorption spectra. All dichroic bands have small, negative Cotton effects and are visible at high optical density of the chromophore. In the legend to Fig. 1 the characteristics of absorbance and dichroic peaks have been collected. Let us first discuss the spectra of enhanced GFP. For GFP two dichroic bands can be observed coinciding with the absorption bands at 490 nm and 395 nm. Although the absorption band arising from state A has a much smaller oscillator strength than the one arising from state B, the rotational strengths of both bands are of comparable magnitude. Similarly, as presented earlier [20], we have analyzed the absorption bands in Gaussian contributions in order to estimate the locations of the electronic transitions of A, B and I bands. We did the same for the CD spectra. For comparison with the peak-normalized optical absorption spectra, all CD spectra have been multiplied by −1 and are peak normalized. In Fig. 2 an example is shown of the decomposition in Gaussian bands for light absorption and CD spectra of GFP on a wavenumber scale. Three main overlapping bands can be observed which can be assigned to the protonated A form (absorbance ca. 409 nm; CD ca. 400 nm), the B–B* transition (absorbance 469 nm; CD 478 nm) and the I–I* transition (absorbance 495 nm; CD 502 nm). When these locations are compared with the locations of the 0,0 transition energies for B and I bands obtained by cryogenic spectral hole-burning [21,22] the agreement is remarkable: B–B*, wild-type GFP 477 nm, S65T mutant 478 nm; I–I*, wild-type 495 nm, S65T 495 nm. The fact that the CD and absorbance band locations are not identical may be due to the fact that the relative rotational strengths of the B and I transitions differ from the relative oscillator strengths. Support for this argument is that the shape of the CD and absorption bands is different at the low energy side of the spectrum, showing a more pronounced peak in the CD spectrum. If we fix the positions of the B and I electronic bands to the ones obtained from the absorption bands in order to resolve both Gaussian bands in the CD spectrum, an equally acceptable fit is obtained as judged from the residuals (results not shown), but with the I band indeed having a much larger rotational strength than the B band. The reversed procedure for fitting the absorption spectrum using fixed CD peak locations did not result in acceptable fits (results not shown). Another striking point of attention is the overlap of the individual bands in the absorption spectra. In previous work by our group [24,56,57] we noticed that the fluorescence decay of GFP was not homogeneous upon excitation at 460–480 nm. In this excitation wavelength range three optical transitions are simultaneously excited (even a small percentage of the A state, see Fig. 2A) and it is therefore not surprising to observe the fluorescence lifetimes of different species yielding a heterogeneous fluorescence decay pattern. Indeed, by exciting at the red edge of the absorption band of GFP, Cotlet et al. [20] were able to excite only the I band and obtained a single fluorescence lifetime of 3.3 ns.

The relative Gaussian areas of all sub-bands, both from absorbance (positive) and CD (negative), for the FPs have been collected in Fig. 2C. A general inspection of CD and absorbance spectra of all FPs shows that only the normalized absorption and CD spectra of BFP and CFP are perfectly overlapping. The two resolved Gaussian bands of BFP and CFP have a vibronic signature. The vibrational frequency derived from the band positions is for BFP 1561 cm^{-1} and for CFP 1157 cm^{-1}. The chromophore of BFP contains a histidine moiety instead of phenolate, while the chromophore of CFP contains the heavier indole residue. The CD spectrum of YFP is distinctly narrower (full width at half maximum (FWHM) 17 nm) than its absorption spectrum (FWHM 26 nm). As with GFP a tentative explanation is that the relative rotational strengths of the bands assigned to the B and I electronic transitions are different from the relative oscillator strengths of these transitions in YFP. Indeed, using the same

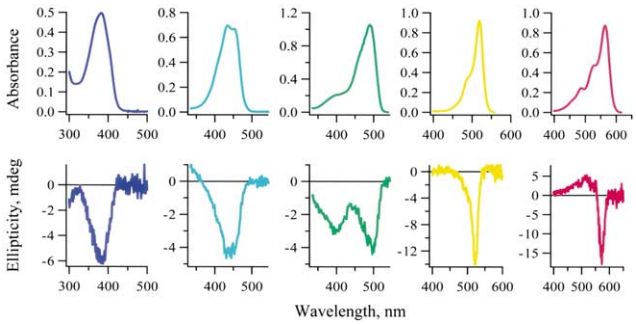

Fig. 1. Light absorption spectra (upper panel) and CD spectra (lower panel) of FPs in 0.1 M Tris–HCl buffer pH 8.0 containing 100 mM potassium chloride. The spectra are shown from left to right for BFP, CFP, GFP, YFP and DsRed, respectively. Using the relationship ellipticity $\theta = 3300\Delta\varepsilon$ ($\Delta\varepsilon$ is the molecular extinction coefficient difference for left and right circularly polarized light) and taking extinction coefficients listed in reference [2] for BFP (22 000 M^{-1} cm^{-1}), CFP (32 500 M^{-1} cm^{-1}), GFP (56 000 M^{-1} cm^{-1}) and YFP (62 000 M^{-1} cm^{-1}) and in reference [42] for DsRed (75 000 M^{-1} cm^{-1}), the wavelengths of maximal absorbance (A_{max}) and minimal $\Delta\varepsilon$ and their values are given for all FPs. BFP: $A_{max} = 382$ nm, $\Delta\varepsilon$ (385 nm) = −79 M^{-1} cm^{-1}; CFP: $A_{max} = 434$ nm, $\Delta\varepsilon$ (432 nm) = −66 M^{-1} cm^{-1}; GFP: $A_{max} = 490$ nm, $\Delta\varepsilon$ (497 nm) = −67 M^{-1} cm^{-1}; YFP: $A_{max} = 518$ nm, $\Delta\varepsilon$ (523 nm) = −293 M^{-1} cm^{-1}; DsRed: $A_{max} = 564$ nm, $\Delta\varepsilon$ (570 nm) = −373 M^{-1} cm^{-1}.

procedure described above for the resolution of Gaussian bands of the GFP-CD spectrum resulted in a much larger rotational strength of the B state as compared to that of the I state (note that the order of both bands is reversed in YFP as compared to GFP). However, since the molecular structure of the yellow chromophore involves two phenyl rings in a π–π stacking conformation some excitonic character of the CD band cannot be ruled out. Again, the location of both bands shows a remarkable similarity with the results obtained by Creemers et al. [22]. The spectral hole-burning experiments on YFP yielded for the B–B* transition 525 nm and for the I–I* transition 496 nm.

The CD spectrum of DsRed is completely different from its absorption spectrum. The CD spectrum has an excitonic signature in which the negative band with a minimum at 570 nm becomes positive at lower wavelengths, coinciding with the shoulder at around 528 nm. The CD minimum of 570 nm does not coincide with the light absorption maximum of 564 nm, but is located at the inflection point of the low-wavelength edge of the absorption band. Recently, it has been pointed out by Garcia-Parajo et al. [49] that in the DsRed tetramer containing four strongly absorbing chromophores the molecular exciton model of energy transfer may be valid. In [58] the CD spectrum for a dimer of certain geometry has been calculated. There will be two CD exciton bands in the dimer of opposite signs and separated into energy by a narrow exciton splitting. The two exciton bands arise from three contributions, namely a one-electron term, electric-magnetic coupling and a coupled-oscillator (or exciton) term, the latter often being the most dominant one. In principle, one can estimate the exciton splitting from the separation between negative and positive bands. In DsRed there are four chromophoric groups in a given geometry and the situation is more complicated. An order of magnitude of the exciton splitting can be obtained by taking the energy difference between the absorption maximum (564 nm, 17 730 cm^{-1}) and the minimum CD band (570 nm, 17 543 cm^{-1}), yielding 187 cm^{-1}. This is a non-negligible quantity and implies relatively strong coupling between the chromophores.

3.2. Far-UV CD

The experimental and fitted far-UV CD spectra of GFP are presented in Fig. 3. The fitted spectrum is obtained after analysis using the CONTIN program. The results of the analysis in secondary structure components are given in the legend in Fig. 3 for the set of 16 protein standards used by Provencher and Glöckner [54]. When the data are analyzed using a second set which additionally has four denatured proteins as models for unordered structure [59], the only change is an increase in

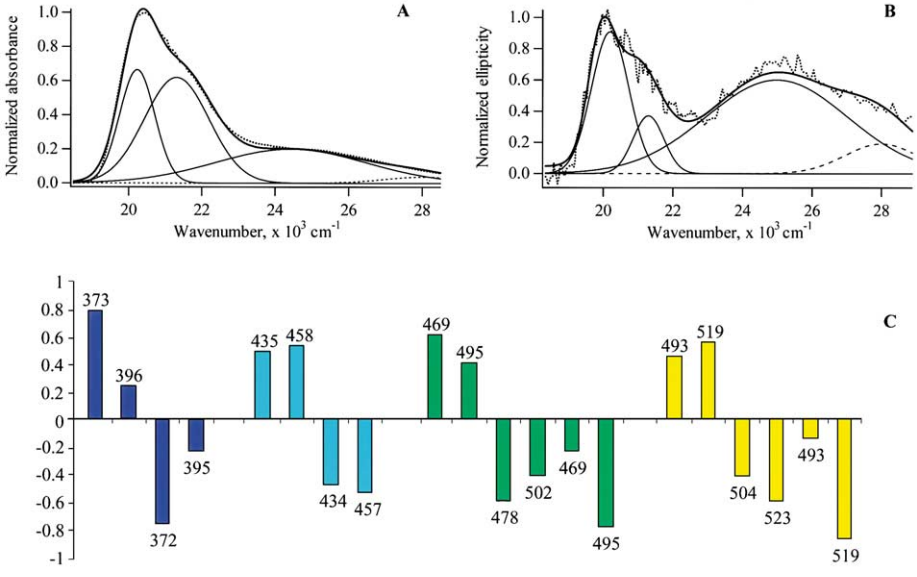

Fig. 2. Decomposition of the absorption spectrum (A) and CD spectrum (B) of GFP in Gaussian bands in order to get an estimate of the spectral positions of the different transitions. Spectra are presented on a wave number scale and are normalized for clarity. In panel C all results have been collected for four different FPs. The relative area of the Gaussian bands (relative intensity) and the band positions have been given both for absorption spectra (positive bars) and for CD spectra (negative bars). In the case of CD spectra of GFP and YFP, the extra bars show the relative intensities when the band positions were fixed to those of the absorption spectra.

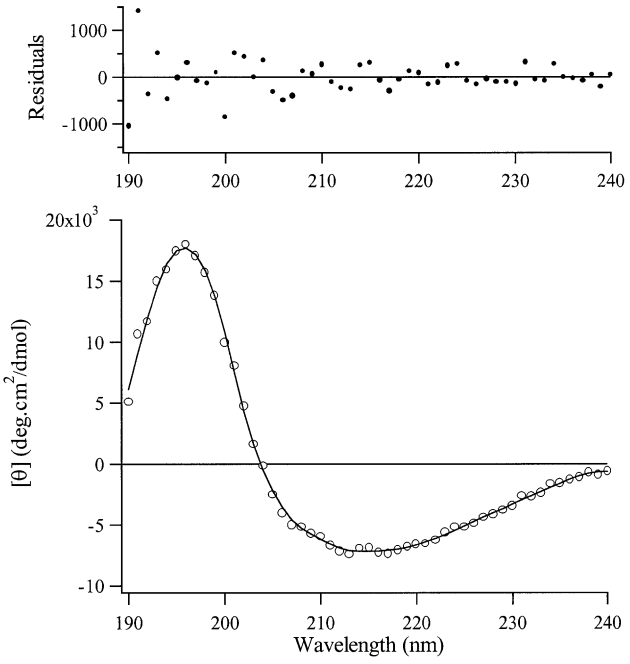

Fig. 3. Experimental (○) and fitted (solid line) far-UV CD spectra of GFP at pH 7.5. The secondary structure content recovered by the CONTIN program is as follows: α-helix 20 ± 1%, β-sheet 52 ± 2%, β-turn 16 ± 1% and remainder 13 ± 1%. The results are based on three different experiments, in which the recovered values are averaged. Residuals are presented in the top panel.

β-sheet content (60 ± 8%) at the expense of non-organized structure (5 ± 4%). As pointed out by Greenfield [55], the results are indeed somewhat dependent on the choice of proteins in the data base of standard proteins. Nonetheless the recovered high content of β-sheet is in complete agreement with the prominent β-barrel structure present in the protein crystal. Therefore we surmise that GFP is representative for a standard protein of high β-sheet content in secondary structure determinations of proteins using CD.

4. Conclusion

CD spectra of FPs provide additional features of the optical transitions of FPs. The fine structure observed in the absorbance and CD spectra of BFP and CFP is indicative of vibrational transitions superimposed on the electronic bands. The absorbance and CD spectra of GFP and YFP reveal that the relative oscillator strengths of B and I transitions do not scale with the relative rotational strengths of both transitions. The CD spectrum of DsRed has an excitonic character, implying coupling of the chromophores in the tetramer.

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