

Origin of the 701 nm fluorescence emission of the Lhca2 subunit of higher plant Photosystem I

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Abbreviations:

CD, circular dichroism; Chl, chlorophyll; β -DM, β -dodecylglucomaltoside; FWHM, full width half maximum ; LD, linear dichroism; Lhc, light harvesting complex; PSI(II), Photosystem I(II); WT, wild type

Summary

Photosystem I of higher plants is characterized by red-shifted spectral forms deriving from chlorophyll chromophores. Each of the four Lhca1-4 subunits exhibits a specific fluorescence emission spectrum, peaking at 688, 701, 725 and 733 nm respectively. Recent analysis revealed the role of chlorophyll-chlorophyll interactions of the red forms in Lhca3 and Lhca4, while the basis for the fluorescence emission at 701 nm in Lhca2 is not clear yet. We report a detailed characterization of the Lhca2 subunit using molecular biology, biochemistry and spectroscopy, and show that the 701 nm emission form originates from a broad absorption band at 690 nm.

Spectroscopy on recombinant mutant proteins assesses that this band represents the low energy form of an excitonic interaction involving two Chl a molecules bound to sites A5 and B5, the same protein domains previously identified for Lhca3 and Lhca4. The resulting emission is however substantially shifted to higher energies. These results are discussed on the basis of the structural information that recently became available from X-ray crystallography (Ben Shem, A., Frolow, F., and Nelson, N. (2003) *Nature* **426**, 630-635). We suggest that, within the Lhca subfamily, spectroscopic properties of chromophores are modulated by the strength of the excitonic coupling between the chromophores A5 and B5, thus yielding fluorescence emission spanning a large wavelength interval. It is concluded that the interchromophore distance rather than the transition

energy of the individual chromophores or the orientation of transition vectors represents the critical factor in determining the excitonic coupling in Lhca pigment-protein complexes.

Introduction

Photosystems I and II have a common general organization including a core complex moiety, binding Chl a and β -carotene, and an outer antenna moiety, binding Chl a, Chl b and xanthophylls. Nonetheless, their spectroscopic properties are substantially different. Both compartments of the PSI supercomplex are enriched in red-shifted chlorophyll absorption forms, while those of PSII are not. This property of PSI results in a preferential light harvesting in the near-infrared spectral region (>700 nm) and causes the so-called “Emerson effect” that opened the way to the discovery of the two photosystems (1). In green plants, LHCI, the outer antenna of PSI, is the most red-forms enriched compartment and is composed of four different complexes, the products of the genes Lhca1-4 (2). These complexes are located on one side of the core complex (3;4) and can be isolated as dimers (5). The Lhca proteins belong to the Lhc multigenic family and they share high sequence homology with the antenna complexes of PSII (6). Despite these strong similarities, the spectroscopic properties of Lhca and Lhcb complexes are very different. In particular, the emission spectrum of LHCI peaks at 733 nm, more than 50 nm red-shifted as compared to the emission spectra of the antenna system of PSII (LHCII) (7). In vitro reconstitution has shown that the red shifted Chls are a common characteristic of Lhca complexes, although their energies differ strongly in different subunits. Emission forms at 701-2 nm have been found associated to Lhca1 and Lhca2, while 725 and 733 nm emissions were reported for Lhca3 and Lhca4 respectively (5;8-11). It has been shown that the red emission of Lhca3 and Lhca4 originates from the low energy absorption band of an excitonic interaction involving Chl a in sites A5 and, probably, B5 (12) while the difference in energy is associated with the nature of the Chl A5 ligand,

since the substitution of Asn in Lhca3 and Lhca4 by His, the usual ligand for Chl A5 in Lhc complexes, leads to the loss of the red spectral forms (12). In the present work we analyze in detail Lhca2 with the aim of understanding the origin of the 701 nm emission which dominates the fluorescence spectrum at low temperature. It should be noticed that Lhca bears His as a ligand for Chl A5 and yet its fluorescence emission is red shifted by 20 nm with respect to the highly homologous subunit Lhcb6 (CP24), and other Lhcb proteins, thus opening the question whether a different mechanism of modulation for the physico-chemical properties of chromophores is at work in Lhca2. By performing a detailed analysis by biochemical and spectroscopic methods on WT and mutant Lhca recombinant proteins we conclude that the fluorescence emission band at 701 nm derives from excitonic coupling between chromophores A5 and B5, in spite of the presence of a His ligand for Chl A5. These results are discussed on the basis of the structural information that recently became available from X-ray crystallography (1;13). We suggest that in green plant LHCI differences in interchromophore distances rather than in transition energies of the individual chromophores of the interacting pair or in the orientation of their transition vectors represent the critical factor in determining excitonic coupling.

Experimental procedure

DNA constructions and isolation of overexpressed Lhca apoproteins from bacteria. cDNAs of Lhca2 from *Arabidopsis thaliana* (11) were mutated with QuickChange© Site directed Mutagenesis Kit, by Stratagene©. WT and mutants apoproteins were isolated from the BL21 strain of *E. coli* transformed with these constructs following a protocol previously described (14;15).

Reconstitution and purification of protein-pigment complexes were performed as described in (16) with the following modifications: the reconstitution mixture contained 420 μg of apoprotein, 240 μg of chlorophylls and 60 μg of carotenoids in total 1.1 ml. The Chl a/b ratio of the pigment mixture was 4.0. The pigments used were purified from spinach thylakoids.

Protein and pigment concentration. HPLC analysis was as in (17). Chlorophyll to carotenoid ratio and Chl a/b ratio was independently measured by fitting the spectrum of acetone extracts with the spectra of individual purified pigments in acetone 80% (18).

Spectroscopy. The absorption spectra at RT and 77K were recorded using a SLM-Aminco DK2000 spectrophotometer, in 10 mM Hepes pH 7.5, 20% glycerol (60% at LT) and 0.06% β -DM.

Wavelength sampling step was 0.4 nm; scan rate: 100 nm/min; optical path length, 1 cm.

The absorption spectra at 4K were measured using a home-build spectrophotometer at OD 0.5 in the same buffer as described above, but with 67% glycerol.

Fluorescence emission spectra at 77K were measured using a Jasco FP-777 spectrofluorimeter and corrected for the instrumental response. The samples were excited at 440, 475 and 500 nm. The spectral bandwidth was 5 nm (excitation) and 3 nm (emission). The chlorophyll concentration was about 0.02 $\mu\text{g}/\text{ml}$ in 60% glycerol and 0.03% β -DM. The spectra between 130 and 4K of the WT were measured with a 0.5 m imaging spectrograph (Chromex 500IS) and a CCD camera (Chromex Chromcam I). For broad band excitation a tungsten halogen lamp (Oriel) was used with band pass filters transmitting at 420, 475 and 506 nm. For narrow band excitation a dye laser (Coherent CR599) was used which was pumped by an argon ion laser (Coherent Inova 310). The optical bandwidth of the laser was 0.6 nm and the power was kept at 0.15 mW/cm². The spectra were

corrected for the wavelength dependence of the detection system.

CD spectra were measured at 10°C on a Jasco 600 spectropolarimeter. The wavelength sampling step was 0.5 nm, the scan rate 100 nm/min and spectra were recorded with 8 accumulations. The OD of the samples was 1 at the maximum in the Qy transition for all complexes and the samples were in the same solution as described for the absorption measurements. All spectra presented were normalized to the polypeptide concentration based on the Chl binding stoichiometry.

LD spectra were measured as described in (19) using samples oriented by the polyacrylamide gel squeezing technique.

Results

Analysis of Lhca2-WT complex

Lhca2 WT was obtained by overexpression of the apoprotein in *E.coli* and reconstitution in vitro with pigments. The complex was purified from excess pigment and unfolded apoprotein by sucrose gradient ultracentrifugation, anion exchange chromatography and glycerol gradient ultracentrifugation, and was obtained in monomeric form, as previously described (11).

Absorption

The absorption spectrum of Lhca2 at 4K shows its maximum in the Chl Qy region at 679.5 nm,

while in the Chl b region two local maxima can be detected at 644 and 652 nm (Fig. 1). The second derivative analysis allows resolution of several absorption forms: three Chl b at 643, 651 and 653 nm and four Chl a at 661, 672, 680.5 and 688.5 nm. In the blue region, the minimum at 497 nm is associated to the red most peak ($S_0 > S_{2,0}$ transition) of the carotenoids, while those at 480 and 471 nm may represent the Soret bands of two Chl b spectral forms. (suggested location figure 1)

Fluorescence measurements

Fluorescence emission spectra of Lhca2 were measured at different temperatures upon excitation in the blue spectral region (Fig. 2). Two emission forms at 688 and 701 nm can be detected. The latter dominates the spectrum at 4K, but the 688 nm form is still present, indicating that the energy transfer between these two forms is not complete, thus suggesting some kind of heterogeneity in the sample. (suggested location figure 2)

In order to get information on the origin of the two fluorescence emissions, fluorescence emission spectra upon selective excitation in the Qy band of the complex were measured. A selection of spectra is presented in fig. 3A. The relative contribution of the 688 and 701 nm forms changed as a function of the excitation wavelength. In particular, the 688 nm emission reached its maximum for excitations in the 680-683 nm range, indicating that it originates from the red part of the bulk absorption. For excitation above 685 nm the amplitude of the 688 nm emission band drops considerably and the spectrum is dominated by the low energy emission form.

In fig. 3B the excitation wavelength is plotted versus the maximum of the emission (squares). By

increasing the excitation wavelength from 680 to 685 nm a blue shift of the emission maximum of the red most form to 697 nm (excitation at 685 nm) is observed. This blue shift is due to the contribution to the emission of a larger fraction of “red” pigments absorbing in the blue wing of the inhomogeneous distribution which are selectively excited at this wavelength (20;21). Above 685 nm the emission maximum undergoes a red-shift, exhibiting a linear relation with the excitation wavelength, thus reaching a value of 701 nm for excitation at 690 nm. By exciting in the red-most part of the absorption spectrum a further red-shift of the emission maximum was observed. For excitations within the red tail of the absorption spectrum (around 710 nm) the emission maximum is still broad (340 cm^{-1}) indicating large homogeneous broadening. Moreover, for excitation above 700 nm, site selection does not have a strong influence on the shape and the width of the emission, thus suggesting that the spectrum is dominated by inhomogeneous broadening. (suggested location figure 3)

The site-selected fluorescence data allow also determining the peak wavelength of the absorption form responsible for the 701 nm emission. This value corresponds to the excitation wavelength at which the emission peak coincides with that in spectra obtained from non selective excitation (see (20) for more details). By following this procedure the absorption maximum of the red band is calculated to be approximately at 690 nm, in agreement with the second derivative analysis of the absorption spectrum which showed the red-most absorption form at 688.5 nm (see Fig. 1).

Additional information on the characteristics of the red absorption tail can be obtained by fluorescence anisotropy measurements, which gives an indication about the orientation of the absorbing and emitting dipoles. In figure 3B (circles) the values of the fluorescence anisotropy

detected at 701 nm are reported as a function of the excitation wavelength. It is clear that the values of the anisotropy also depend on the excitation wavelength. Upon excitation around 680 nm the anisotropy value is low and it rises to a maximum of 0.32 for excitation wavelength above 697 nm, a value very similar to that obtained in the analysis of the 702 nm emission form in a native LHCI preparation (7). This result indicates that above this wavelength no depolarization due to energy transfer takes place, thus suggesting that the same band is responsible for the absorption and the emission. The alternative hypothesis of energy transfer between pigments having the same dipole transition orientation is extremely unlikely.

LD spectra

The LD spectrum of Lhca2 shows strong positive contribution in the Chl a Q_y absorption region, with maximum at 679.5 nm and a shoulder around 690 nm (Fig. 4). The signal becomes slightly negative at 667 nm. In the Chl b absorption region, a positive contribution is observed at 653 nm. The reduced LD spectrum (LD/3A) shows the largest value around 690 nm, indicating that the Chl(s) responsible for this absorption forms is(are) oriented with the largest angle with respect to the normal to the membrane plane. A second positive LD value corresponds to the maximum of the absorption spectrum (679.4 nm), while a local minimum is present at 683 nm. (suggested location figure 4)

Localisation of the low energy absorbing form(s) in to the Lhca2 structure

The above measurements strongly indicate that the red-shifted fluorescence signal at 701 nm is associated with an absorption form peaking at 690 nm. In order to identify which chromophore is

responsible for this absorption, mutation analysis at selected Chl-binding residues was performed. Based on previous analysis with Lhca3 and Lhca4 (12), residues coordinating Chl A5, B5 and B6, involved in the low energy absorption forms, were mutated to non-binding residues (i.e. which can not coordinate the central magnesium of the Chls (see table I for details). The apoprotein was expressed in *E. coli* and the complex reconstituted *in vitro* with pigments. (suggested location table D). In all cases stable monomeric complexes were obtained. The pigment content of the complexes is shown in table I.

The decreased Chl /Car ratio observed in all mutants indicates loss of Chls in the Chl binding site mutants and the higher Chl a/b ratio as compared to the WT complex implies that Chl b molecules are preferentially lost. Normalisation to the carotenoid content would indicate loss of 1 Chl in the A5 mutant, 2 Chls in B5 and 1 Chl in B6. However, this stoichiometry may not be correct in the case of mutants A5 and B5, since the corresponding mutants in all other Lhca complexes showed a loss of carotenoid molecules. Moreover, loss of 1 Chl in A5 would indicate that this site has mixed occupancy, which is very unlikely, because the A5 site has been found to accommodate one Chl a molecule in all complexes analysed so far (22-24). We thus suggest that the A5 mutant loses 2 Chls molecules: 1 Chl a and 1 Chl b, as was the case in Lhca1, while a small loss of carotenoids account for the change in Chl/Car ratio. The B5 mutant loses most probably 3 Chl molecules, 2 Chls b and 1 Chl a. The loss of Chl a is consistent with the analysis of the absorption spectrum (see below) which is strongly affected in the Chl a Q_y absorption region.

To check the influence of the mutation on the 701 nm emission form of the complex, fluorescence emission spectra at 77K were measured (figure 5). Mutant A5 and B5 showed emission maximum at 686 nm, thus shifted by 15 nm to the blue as compared to the WT peak, indicating that both

mutations affect the low energy emission form. The fluorescence emission of the B6 mutant was, instead, very similar to that of the WT, showing that mutation at this Chl binding site does not influence the chromophore(s) responsible for the 701 nm emission. (suggested location figure 5)

The absorption spectra of the three mutants and the WT at 77K are presented in figure 6A and the second derivative of the spectra are shown in fig 6B.

Mutant A5 was mainly affected in the Chl a region, where the absorption form at 690 nm disappeared. A decrease in the amplitude of the spectrum in the Chl b region could also be observed, in agreement with the Chl b loss (see table I). Mutant B5 showed dramatic changes in the absorption spectrum as compared to the WT: not only two Chl b forms (at 642 and 651 nm) were lost, but also a 688 nm absorbing Chl a form. Absorption at 680 nm was also decreased and was partially compensated by the increased signal at 670 nm, possibly due to the presence of unconnected Chls. Mutant B6 was affected mainly in the Chl b region, where the absorption at 642 nm disappeared, an indication that the Chl b accommodated in the B6 site absorbs at this wavelength. Minor changes could also be detected in the Chl a absorption region. Interestingly, the effect of the mutation at site B6 on the red-shifted absorption forms is different in Lhca2 as compared to Lhca1 (24) and Lhca4 (unpublished results). In these homologous proteins, loss of Chl B6 significantly affected the red-shifted emission forms. This difference may be related to the observation that the B6 mutants of Lhca1 and Lhca4 have lost xanthophylls, while Lhca2 did not. The loss of red forms in Lhca1 and Lhca4 upon mutation at site B6 was interpreted as an indirect effect mediated by the carotenoid molecule (24). The different results obtained for the B6 mutant in Lhca2 seem to indicate that the carotenoid organization in the Lhca complexes is different with respect to other Lhca subunits. (suggested location figure 6)

The CD spectra at RT of WT and mutants are reported in fig. 7. Mutant A5 is identical in the Chl b region to the WT but shows strong differences in the Chl a region. Comparison with the WT spectrum indicates that this mutant loses a negative CD signal at 687 nm and a positive one at 672 nm, thus suggesting loss of an excitonic interaction. A strong decrease in the intensity of the CD signal in the Chl b region is observed for both B6 and B5 mutants, thus implying that the Chl b molecules lost in the B5 and B6 mutants participate in pigment-pigment interactions. In particular the comparison of the spectra of the WT with the one of mutant B6 shows that, in the latter, the loss of the negative signal in Chl b region is connected with the loss of a positive contribution in Chl a region (around 670 nm), thus suggesting the presence of a Chl b-Chl a interaction. (suggested location figure 7)

Discussion

The current results show that Lhca2 takes a unique position within the LHCI proteins. Unlike Lhca3 and Lhca4, it does not show strong absorption above 700 nm, but nevertheless its emission is still 22 nm red shifted as compared to the emission maxima of all Lhcb complexes. In the case of Lhca3 and Lhca4 it was shown that the red absorption is associated with the presence of an Asn as ligand for Chl A5 (12). This is not the case of Lhca2 which has an His in this position, a feature shared with all Lhcb proteins that do not show red-shifted Chl forms. We thus proceeded with the analysis of the chromophore organization in Lhca2 and identified the absorption band responsible for the 701 nm emission band, with the aim of understanding the mechanism which allows modulation of the physico-chemical properties of chlorophyll ligands.

Lhca2 has been shown to have the emission maximum at 701 nm (at 4K). A second emission form was also detected at 688 nm in the 4K spectrum, thus suggesting heterogeneity in the sample. Several sources of spectroscopic heterogeneity have been reported for Lhc proteins, including phosphorylation-induced spectral changes in CP29 (25), presence of multiple gene products in the LHCII preparations with different spectral features (26), and conformational changes induced by zeaxanthin binding to site L2 (27;28). In the present work, a recombinant Lhca2 preparation was analyzed which was the product of a single gene, did not contain zeaxanthin, was monomeric as detected by sucrose gradient ultracentrifugation and was not phosphorylated. We conclude that the persistence of the two fluorescence emission bands is the product of a previously unrecognized source of molecular heterogeneity which is reflected in the establishment or not of the molecular feature responsible for the 701 nm emission. Two hypotheses can be proposed for explanation of these data: either the Lhca2 protein is present into two conformations in which the two interacting chromophores are set at different distances and/or different orientations with respect to each other, or one of the two sites can be occupied by either Chl a or Chl b. The difference in transition energies between Chl a and Chl b and the difference in the orientation of the transition vectors (29) would make the observable spectral shift of a Chl a/Chl b interaction much smaller with respect to that of a Chl a/Chl a interaction. The likelihood of these hypotheses will be discussed below. Site-selected fluorescence measurements indicate that the 689 nm emission originates from bulk Chls absorbing around 682 nm, while the 701 nm emission form is associated with a Chl absorption form at 690 nm. This result is confirmed by the mutation analysis which showed that both mutants losing the 701 nm emission also lack the 690 nm absorption form.

Spectral characteristics of the 690 nm absorption form

In order to get more details on the 690 nm absorption form of Lhca2, the absorption spectrum of the WT complex was described in terms of Gaussian forms, using the values obtained by second derivative analysis as starting parameters (fig. 8). Three Chl b bands were needed to describe the region between 640 and 660 nm, namely at 642, 649 and 655 nm. The red part of the Qy Chl a absorption region was described with two Gaussians at 686 and 690 nm, with the latter showing a broadened spectrum. An oscillation strength corresponding to the absorption of 0.85 Chl a molecules is associated to this band. (suggested location figure 8)

This description is in good agreement with the fluorescence data: the red most band, which is responsible for the 701 nm emission, absorbs at 690 nm, as suggested by the site-selected fluorescence measurements, and a second band, peaking at 686 nm, is needed for description of the red absorption tail. The absorption of this second band fades to zero at around 697 nm, in agreement with the anisotropy measurements which suggest that only above 697 nm excitation energy is exclusively absorbed by the red-most pigment while the 686 nm band is less broadened (8 nm FWHM) thus suggesting this absorption derives from a monomeric pigment.

The energetic separation between the absorption maximum and the emission peak represents the Stokes shift. For the 690 nm absorption form, which emits at 701 nm, the Stokes shift is 11 nm (240 cm^{-1}). This is larger than observed in Lhcb complexes (where it is around 2 nm). From the Stokes shift a value of the optical reorganization energy (Sv) can be obtained, which corresponds to 120 cm^{-1} (Stokes shift = 2 Sv.).

The characteristics of the absorption band broadening can yield important information on the

chromophore protein environment. The analysis of the WT minus mutant difference spectra indicates that at 77K the FWHM of the band is around 400 cm^{-1} . At this temperature the homogeneous broadening of the band can be described as

$$\text{FWHM}_{\text{hom}}^2 = 7.7 \text{ SvT}, \quad \text{with } \text{FWHM}^2 = \text{FWHM}_{\text{hom}}^2 + \text{FWHM}_{\text{ihn}}^2$$

Using the value of Sv obtained from the Stokes shift the value for the FWHM_{hom} is 270 cm^{-1} , which gives a value of the inhomogeneous broadening of around 300 cm^{-1} . Such a large value for the inhomogeneous broadening is consistent with data from site-selected fluorescence measurements since the excitation in the red tail did not affect the shape and width of the emission band.

The 690 nm absorption form of Lhca2 is thus characterized by a larger value of both homogeneous and inhomogeneous broadening as compared to the bulk Chl absorption form for which these two values are both around 100 cm^{-1} (30). In this respect the 690 nm band of Lhca2 shows characteristics very similar to the red absorption forms observed in several organisms (20), although its energy is significantly higher.

The origin of the 701 nm emission

Large values of optical reorganization energy are usually associated with the presence of pigment-pigment interactions. It has already been suggested that the red forms of Lhca3 and Lhca4 are the

low energy term of an interaction between two Chl a molecules. Mutational analysis shows that these two chromophores are bound to sites Chl A5 and Chl B5, since mutation at these sites completely abolishes the red emission forms. This conclusion is in agreement with the finding that site A5 binds Chl a in Lhca as well as in any other Lhc protein (22-24), while site B5 either binds Chl a, or has a mixed Chl a –Chl b occupancy as derived from pigment analysis of WT and mutant protein as well as their spectroscopic analysis. We conclude that the low energy spectral forms originate from the same protein domain in all Lhca complexes and involves Chls A5 and B5. Nevertheless, the energy level of these red-shifted transitions is modulated to a different extent in each Lhca subunit. (suggested location figure 9)

The presence of pigment-pigment interaction inducing the absorption at 688-690 nm of Lhca2 is supported by the comparison of the CD spectra of WT and A5 mutant, which lacks a negative contribution around 687 nm and a positive one at 672 nm (values at RT). Similar components can be detected in the WT minus A5 absorption and LD difference spectra (fig. 9). In both cases two absorptions, at 688-689 nm and at 672-674 nm, can be detected, representing Chl forms lost by effect of the mutation at site A5 and having positive LD spectra. These data can be interpreted as the loss of an excitonic interaction having the low energy band at 688-690 nm and the high energy band at 672-674 nm, as the effect of A5 mutation. To describe both difference spectra a negative band peaking at 679 nm is required. This band is present in the mutant but not in the WT and shows a positive LD signal (Fig 6). Considering that all spectroscopic techniques consistently indicate that the interaction leading to the 690 nm absorption involves two Chl a molecules, and that in the A5 mutant only one Chl a molecule is lost, the gain in the absorption can be interpreted as the contribution of the non-interacting monomer which is left alone in the A5

mutant. Based on the mutation analysis, which suggested that the two interacting Chls are accommodated in sites A5 and B5, we conclude that the “new” 679 nm form detected in WT minus A5 difference spectra represents the absorption of the monomeric Chl a in site B5. This is in agreement with the analysis of the homologous protein CP29 (22) showing that a non-interacting Chl a absorbing at 679 nm is bound to site B5.

From the energetic distance between the two bands in the CD spectrum and considering the two monomers as isoenergetic, a value of 150 cm^{-1} for the interaction energy can be calculated (notice that a very similar value is obtained considering the absorption of monomeric A5 at 675 nm as was the case in all Lhc complexes analysed so far- 22;23). This value is half of the value observed for the interaction energy in Lhca3 and Lhca4 (manuscript in preparation).

Once concluded that in all Lhca complexes the red form originates from interaction between Chls a in sites A5 and B5, it is interesting to investigate the origin of the large difference in interaction energy between the complexes, which is reflected in a difference of around 30 nm between the emission maxima of Lhca2 and Lhca4. Two major factors control the amplitude of the interaction energy: the distance between the chromophores and the relative orientation of their dipole moment transition (the geometric factor k). ($V_{1,2} = 5.5k \mu_1 \mu_2 / R^3$, where R is the centre to centre distance between the chromophores and μ are the dipole moment of the Chls). The analysis of the LD spectra of the complexes indicates that the red most absorption form of both Lhca4 and Lhca2 forms the largest angle with the normal to the membrane plane (12, this work). Although small differences in the orientation between the Chls in the two complexes can not be excluded, we can assume, in first approximation, a similar geometric factor in the two complexes. On this basis,

the difference in interaction energy can be explained by considering that the distance between the two interacting Chls is around 2 Å larger in Lhca2 compared to Lhca4 by applying equation $V = 90k/R^3$. Recently, the structure of PSI-LHCI complex has been elucidated at 4.4 Å resolution and the position of the bound Chl defined. The center to center distance between Chl A5 and B5 in Lhca4 has been shown to be 7.9 Å, while in Lhca2 the value is 10.4 Å (3). This is consistent with the proposal that in Lhca4 the presence of the Asn as a ligand for Chl A5 allows a shorter distance between the two interacting Chls as compared to the complexes which have His for Chl A5 ligation (12).

Comparison with LHCII

Previous work with Lhca3 and Lhca4 has highlighted the importance of the presence of an Asn as a ligand for Chl A5 for the generation of the red shifted 730 nm fluorescence in Lhca3 and Lhca4, the only members of the Lhc superfamily holding Asn as a Chl A5 ligand. The present work shows that a significant, although lower, level of excitonic interaction can be obtained without the intervention of Asn ligation and yet this interaction is sufficient for yielding the 701 nm emission form of Lhca2. This finding implies that additional structural signatures differentiate the Lhca from Lhcb subfamily as requirement for the chromophore-chromophore interactions in the Helix C domain. This problem can be addressed thanks to the recent elucidation of LHCII structure at high resolution since in Lhcb 1-3, the components of trimeric LHCII, the ligand of Chl A5 is His, as in Lhca2, and yet no 701 fluorescence emission can be detected. Moreover the two structures in this domain are also very similar (3;13) thus allowing detailed comparison. Why LHCII does not have red forms?

Both X-ray structure (13) and mutation analysis (23;31) consistently identify the ligand at site B5 as Chl b. Although the distance and mutual orientation between Chl a A5 and Chl b B5 allows for interaction in LHCII (13), the large energy gap between Chl a and Chl b makes the shift induced in the absorption form very small. In the case of Lhca2, biochemical and spectral analysis are consistent with site B5 having low selectivity and thus allowing for binding of both Chl a and Chl b. This mixed occupancy is crucial for explanation of the heterogeneity in fluorescence emission of Lhca 2 (see fig. 2 and text) leading to the presence of two emission forms in the emission spectrum of Lhca2. We suggest that the blue form (emission at 689 nm) originates from the population of the ensemble having Chl b in site B5, while the 701 nm emission is originating from the population in which site B5 accommodates a Chl a molecule. The crucial question is thus how the selectivity of each Chl binding site is determined in Lhca proteins. The structure of LHCII shows that all but one Chl b present have the formyl group H bonded with protein residues of water molecules. It can thus be speculated that the possibility to form this H-bond affects the affinity of a site for Chl b vs. Chl a, through an energy minimization effect. In LHCII Chl b in site B5 is stabilized by a H-bond with the NH of Gln131 (13). In Lhca2 complex, as well as in all Lhca complexes, this Gln is substituted by a Glu residue. The Glu can not act as H- donor and it can be expected that in this case Chl B5 loses its high affinity for Chl b and thus can also accommodate Chl a.

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Figures legend

Figure 1. Absorption spectrum at 4K of Lhca2-WT. The spectrum is presented together with its second derivative.

Figure 2. Temperature dependence of the fluorescence emission spectrum. Fluorescence emission spectra of Lhca2-WT measured at 4K, 40K, 77K, 100K and 130K. The sample was excited at 506 nm.

Figure 3. Site selected fluorescence. (A) Selection of emission spectra of Lhca2 at 4K, laser excitation at 679, 683, 689, 694 and 700 nm. The spectra were normalised to the maximum of the emission. The sharp peak is largely due to elastic scattering of the laser excitation light. (B) emission and absorption characteristics of Lhca2-WT complex at 4K. Closed squares: dependence of the emission maximum as a function of the excitation wavelength. Open circles: value of the anisotropy of the emission detected at 701 nm. Line: tail of the absorption spectrum.

Figure 4. Linear dichroism. LD (solid) and LD/3OD (dashed) spectra of Lhca2-WT at 10K. The scale is in arbitrary units.

Figure 5. Fluorescence emission spectra at 77K of Lhca2 WT and mutants: Lhca2-WT (dashed), Lhca2-A5 mutant (solid) and Lhca2-B6 mutant (dotted) upon excitation at 500 nm. The spectra were normalised to the maximum of the emission.

Figure 6. Absorption spectra at 77K of Lhca2-WT and mutants. (A) Absorption spectra of Lhca2-WT (solid), Lhca2-A5 (dashed), Lhca2-B5 (dotted) and Lhca2-B6 (dash-dot). The spectra are normalised to the Chl content. (B) second derivative of the spectra reported in A

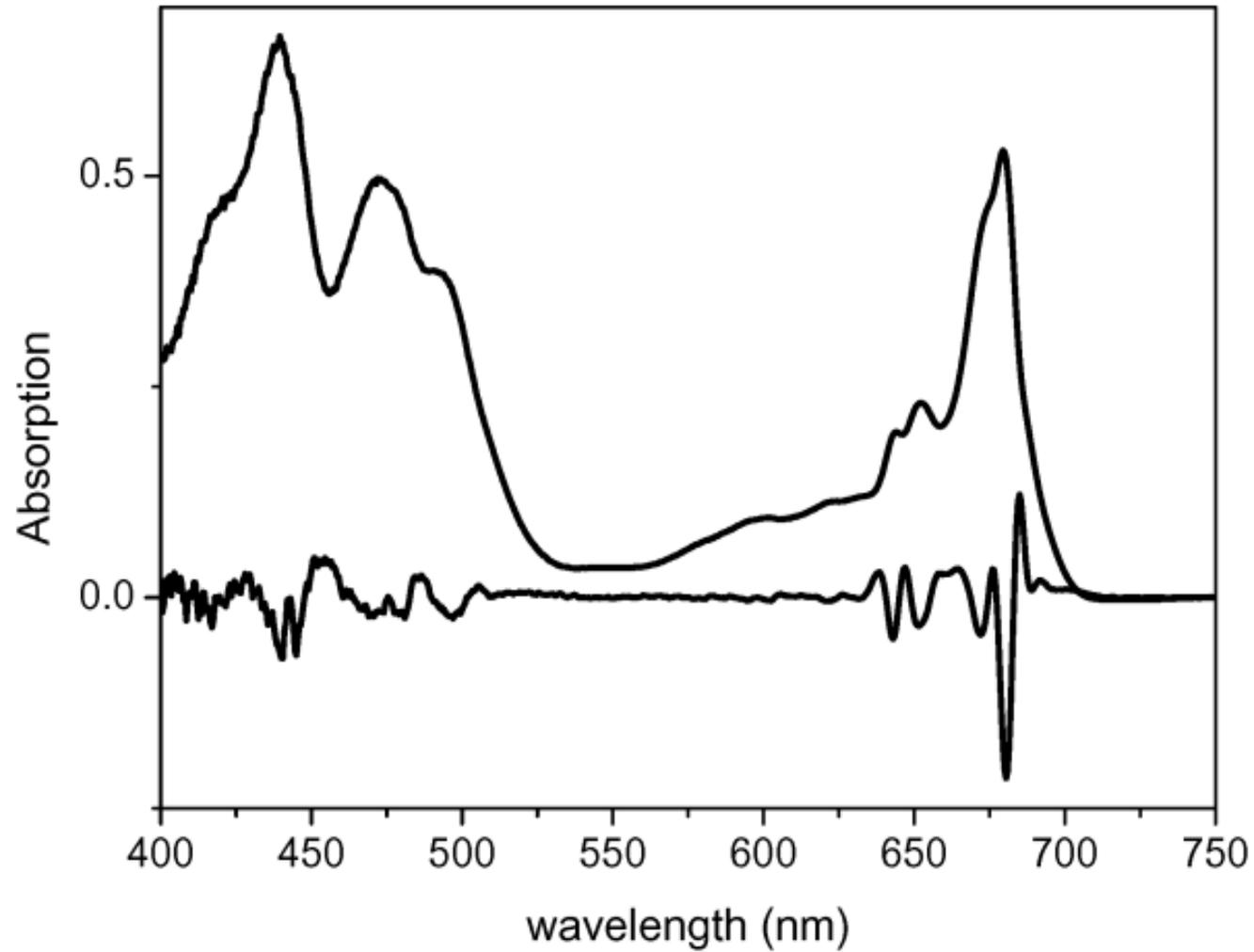
Figure 7. Circular dichroism spectra of Lhca2-WT and mutants at 10 °C. All the spectra are normalised to the absorption (A) Lhca2-WT (dashed), Lhca2-A5 (dotted), difference WT-A5 (solid). (B) Lhca2-WT (dashed), Lhca2-B5 (dotted), Lhca2-B6 (solid).

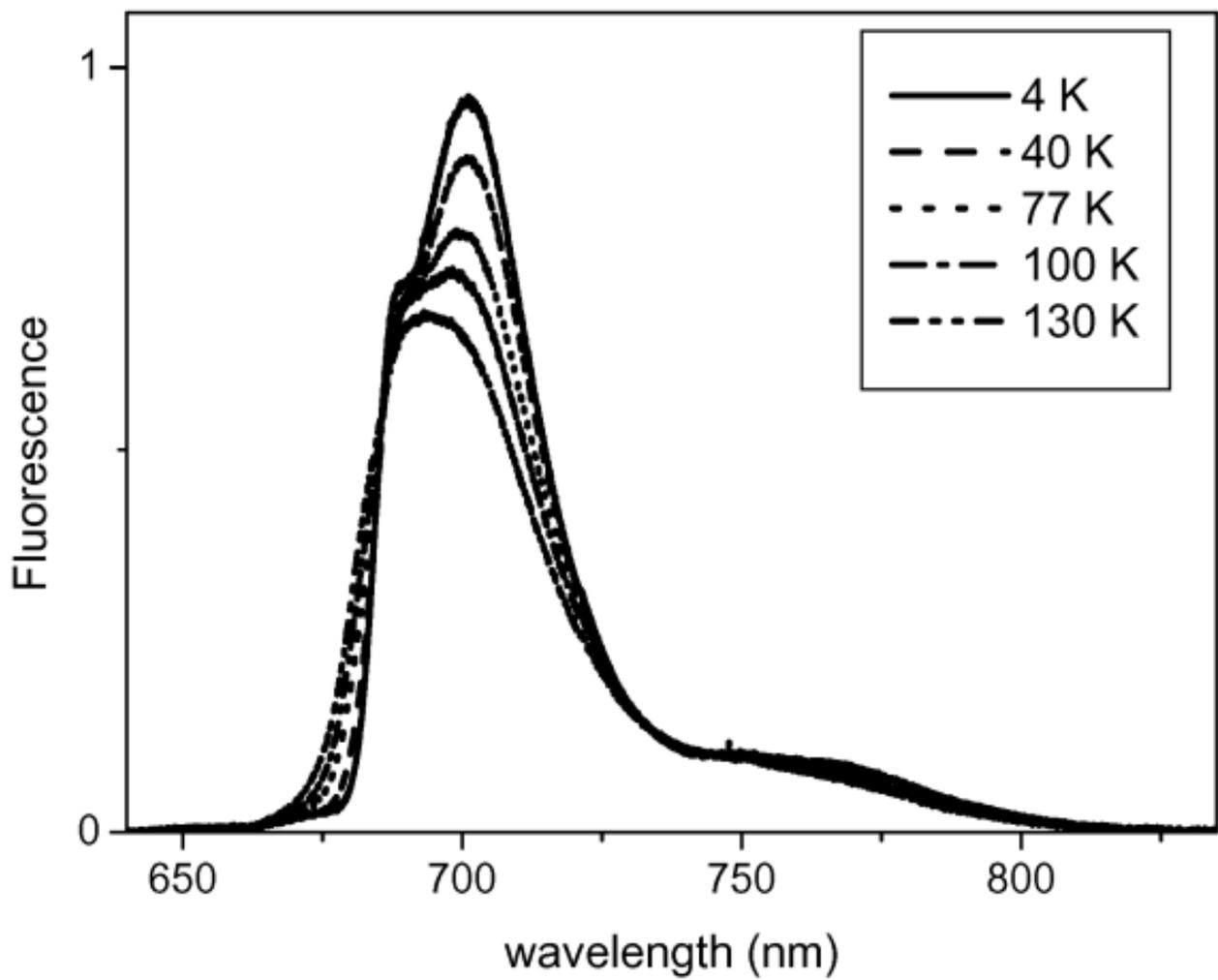
Figure 8. Gaussian description of the absorption spectrum of Lhca2-WT at 4K. Ten Gaussian are used to describe the spectrum.

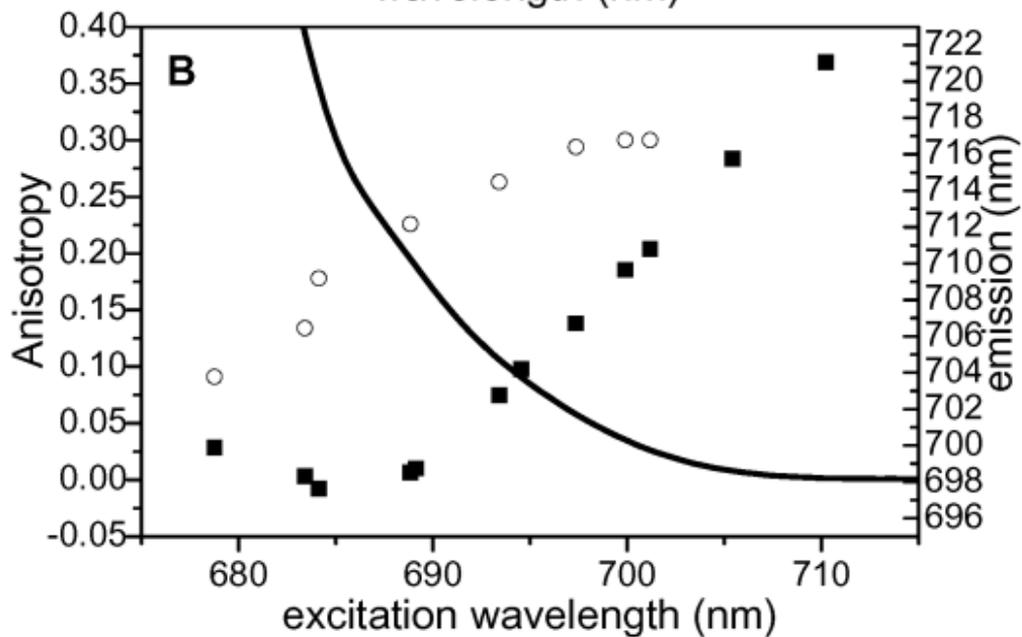
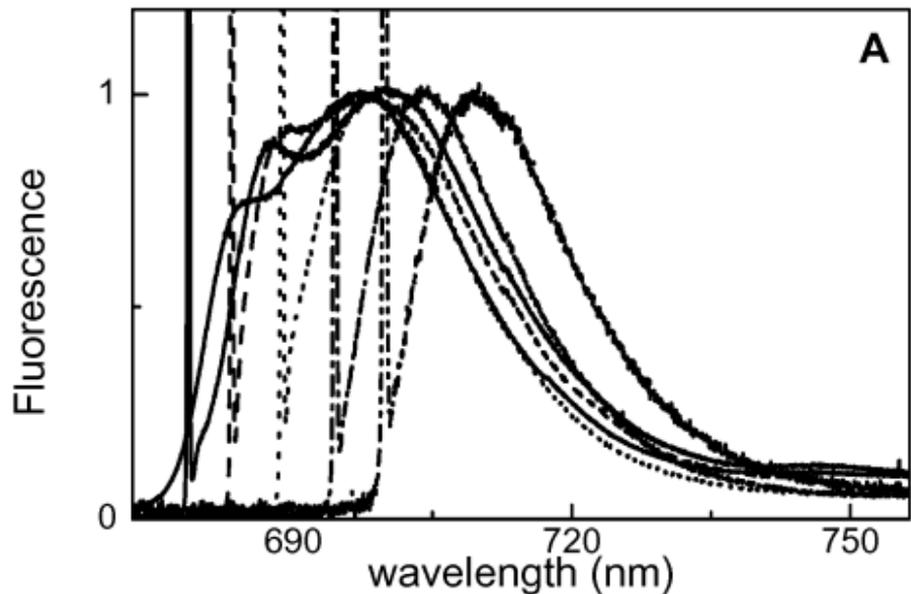
Figure 9. Comparison of the absorption (dashed) and linear dichroism (solid) difference spectra of WT and A5 mutants.

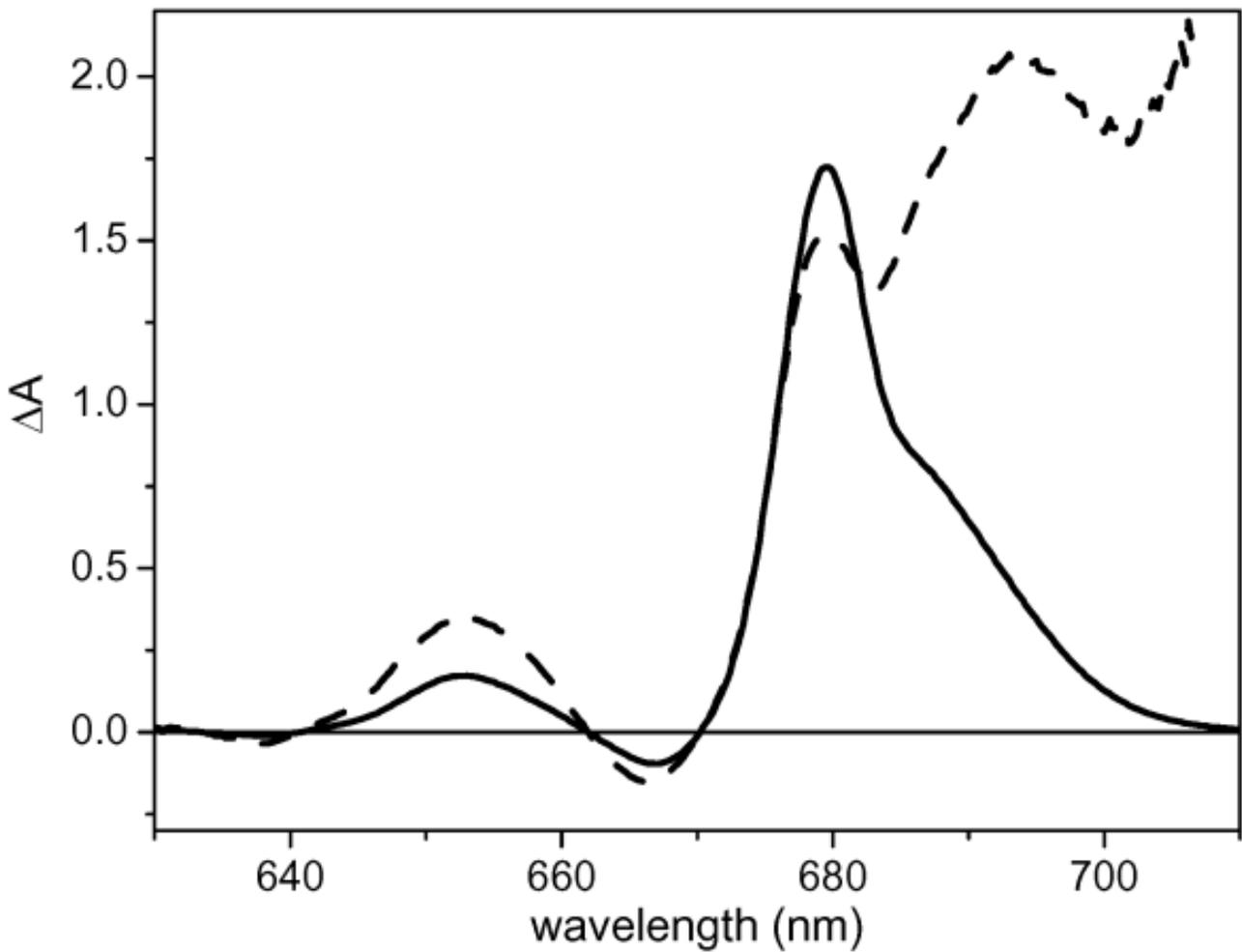
Table I. Pigment composition of the complexes. The amount of carotenoids is calculated for the given Chl stoichiometry. The error in the data is less than 0.1.

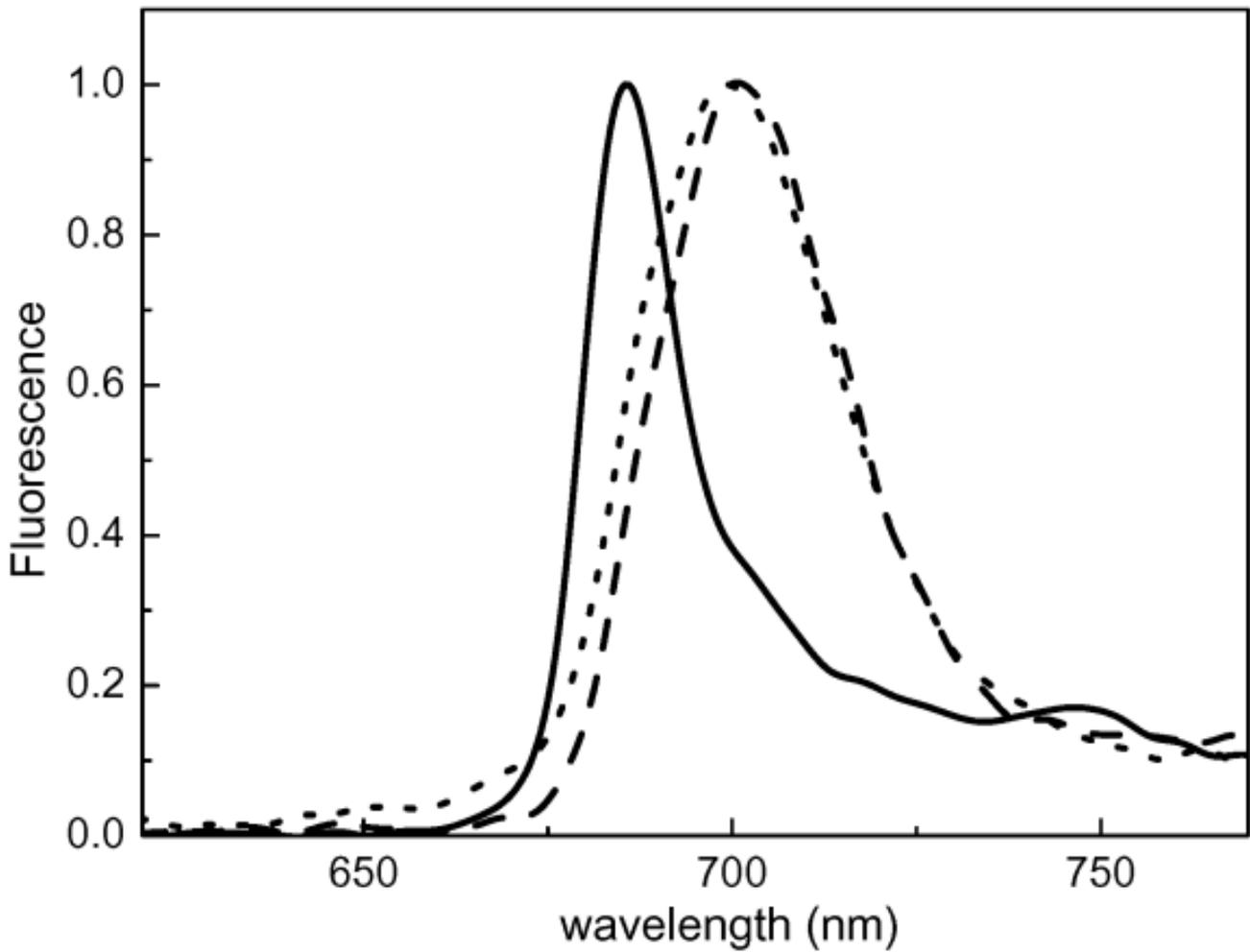
Sample	mutation	Chl a/b	Chl/Car	Chl tot	Lute	viola
rLhca2-WT	-	2.1	5.0	10	1.68	0.32
rLhca2-A5	H52F	2.2	4.5	8	1.41	0.35
rLhca2-B5	E108V/R111L	4.7	4	7	1.44	0.3
rLhca2-B6	E100V	3.1	4.4	9	1.57	0.25

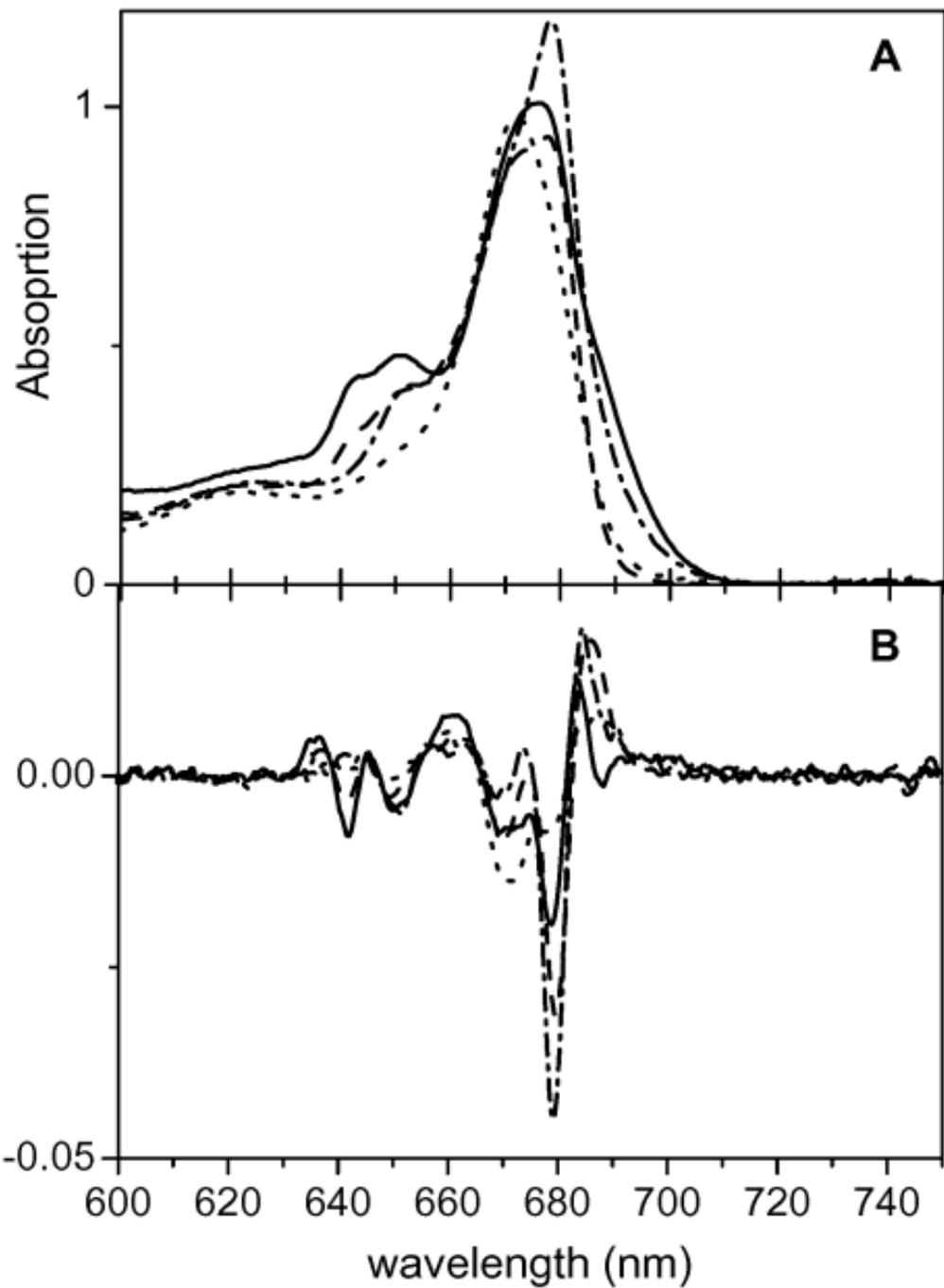


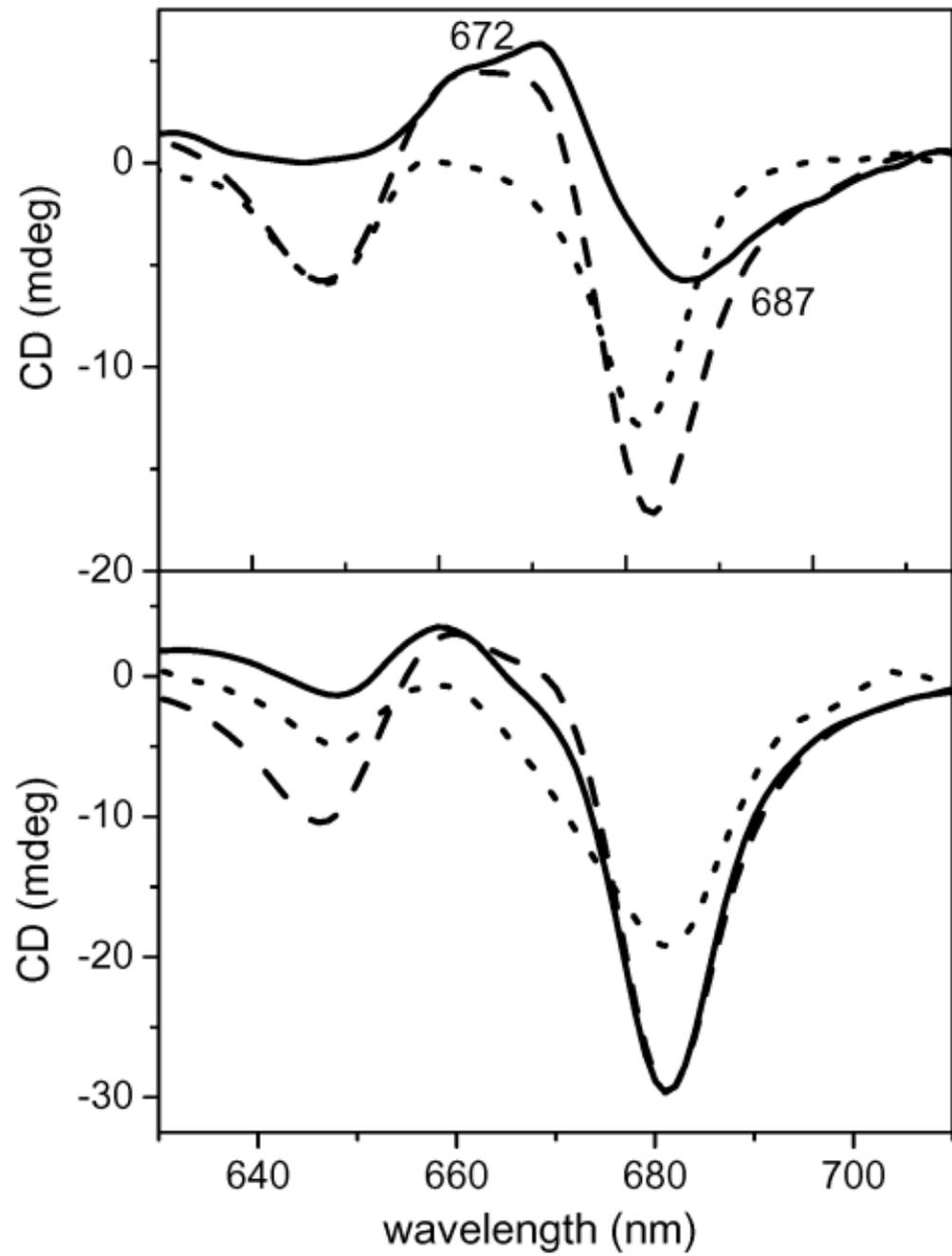


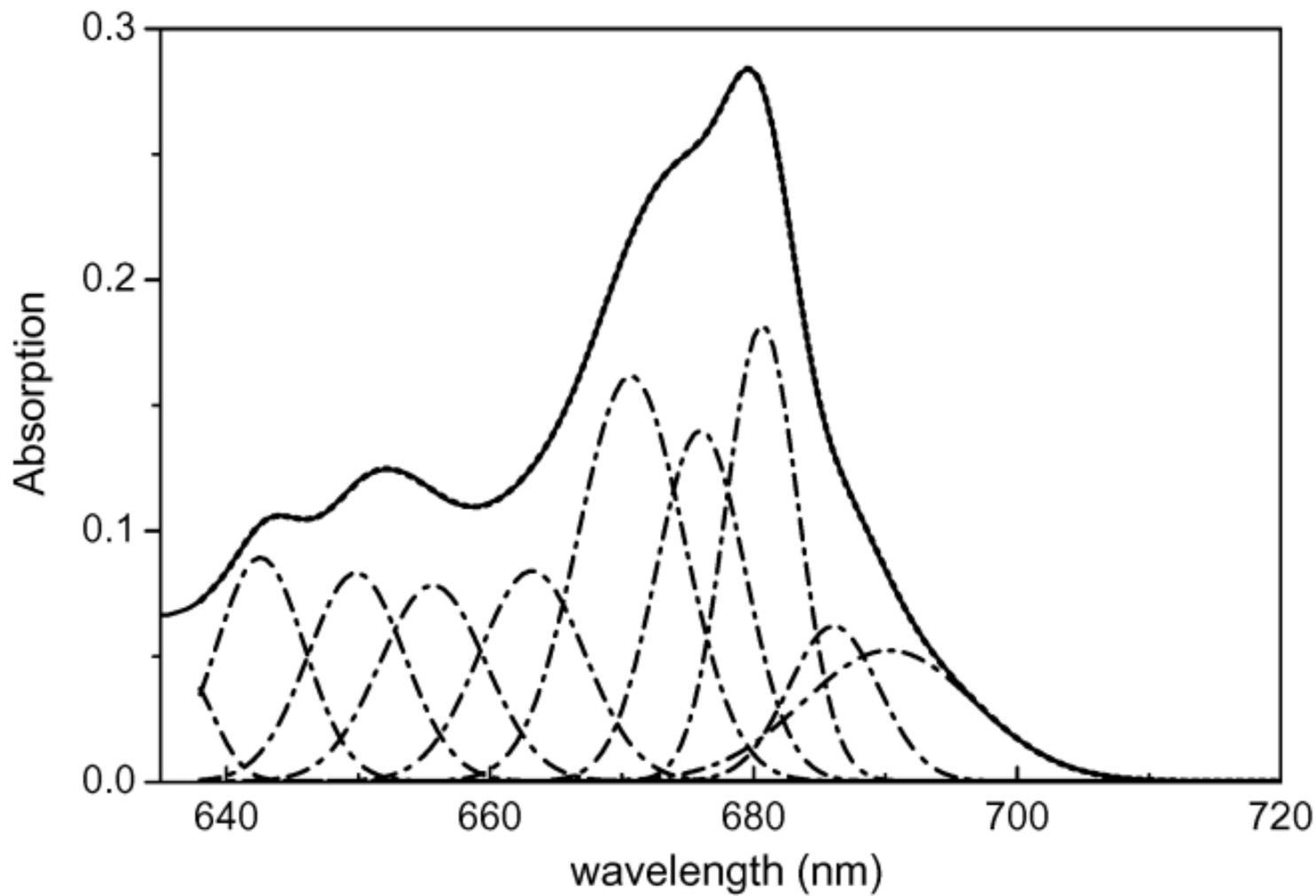


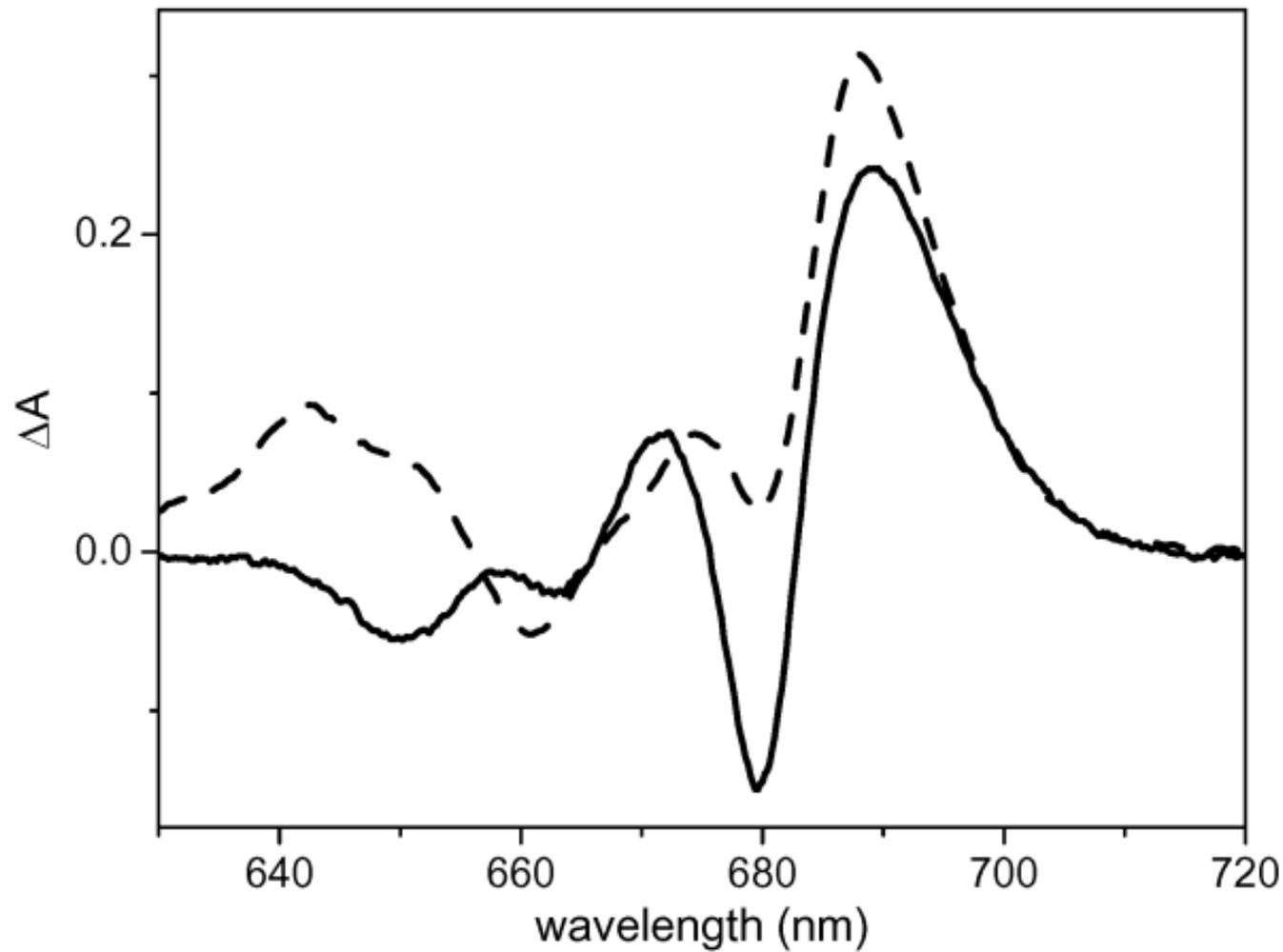












Origin of the 701 nm fluorescence emission of the Lhca2 subunit of higher plant photosystem I

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