

Spectroscopic characterization of the spinach Lhcb4 protein (CP29), a minor light-harvesting complex of photosystem II

Andy Pascal¹, Claudiu Gradinaru², Ulrich Wacker^{3,4}, Erwin Peterman², Florentine Calkoen², Klaus-Dieter Irrgang³, Peter Horton⁴, Gernot Renger³, Rienk van Grondelle², Bruno Robert¹ and Herbert van Amerongen²

¹Section de Biophysique des Protéines et des Membranes, DBCM/CEA and URA 2096/CNRS, CE-Saclay, Gif-sur-Yvette, France;

²Department of Physics and Astronomy and Institute for Molecular Biological Sciences, Vrije Universiteit, Amsterdam, The Netherlands;

³Max-Völmer Institut für Biophysikalische Chemie und Biochimie, Technische Universität Berlin, Germany; ⁴Robert Hill Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, UK

A spectroscopic characterization is presented of the minor photosystem II chlorophyll *a/b*-binding protein CP29 (or the **Lhcb4** protein) from spinach, prepared by a modified form of a published protocol [Henrysson, T., Schroder, W. P., Spangfort, M. & Akerlund, H.-E. (1989) *Biochim. Biophys. Acta* **977**, 301–308]. The isolation procedure represents a quicker, cheaper means of isolating this minor antenna protein to an equally high level of purity to that published previously. The pigment-binding protein shows similarities to other related light-harvesting complexes (LHCs), including the bulk complex LHCIIb but more particularly another minor antenna protein CP26 (**Lhcb5**). It is also, in the main, similar to other preparations of CP29, although some significant differences are discussed. In common with CP26, the protein binds about six chlorophyll *a* and two chlorophyll *b* molecules. Two chlorophyll *b* absorption bands are present at 638 and 650 nm and they are somewhat more pronounced than in a recent report [Giuffra, E., Zucchelli, G., Sandonà, D., Croce, R., Cugini, D., Garlaschi, F.M., Bassi, R. & Jennings, R.C. (1997) *Biochem.* **36**, 12984–12993]. The bands give rise to positive and negative linear dichroism, respectively; both show negative CD bands (cf. bands with similar properties at 637 and 650 nm in CP26). Chlorophyll *a* absorption is dominated by a large contribution at 674 nm which also shows similarities to the major band in LHCIIb and CP26, while (as for CP26) a reduction in absorption around 670 nm is observed relative to the bulk complex. Principal differences from LHCIIb and CP26, and from other CP29 preparations, occur in the carotenoid region.

Keywords: light-harvesting; LHC gene family; photosystem II; polarized spectroscopy.

Photosynthesis is driven by the absorption of light energy by light-harvesting proteins and the transfer of this energy, in the form of an excited state, to other proteins called reaction centres where primary charge separation takes place. In photosystem (PS) II of higher plants and green algae the light-harvesting complexes constituting the inner antenna (CP43 and CP47) bind only chlorophyll *a* and β -carotene, while the outer LHCIIb bind chlorophylls *a* and *b* and a number of xanthophylls (carotenoids with oxygen functions). The latter proteins display a high degree of sequence homology with each other and with the equivalent proteins of PSI (LHCIs), forming part of the LHC multigene family [1,2]. The structure of the bulk LHC of PSII, LHCIIb, which binds 65% of PSII chlorophyll, has been solved to 0.34 nm and an atomic model generated [3]. Three transmembrane helices are evidenced, plus a fourth amphiphilic helix at the membrane–lumen surface, and the positions of at least 12 chlorophylls and two carotenoids are apparent in the structure. Owing to their sequence homology, the other LHC proteins are predicted to form the same basic protein fold, while their

pigment-binding stoichiometries vary significantly. Thus structural information for the other LHC proteins at the pigment level, while incomplete for LHCIIb, is for the moment reliant on spectroscopic techniques.

As well as LHCIIb, there are (at least) three further LHC proteins associated with PSII. These are variously called CP29, CP26 and CP24 [4], or LHCIIa, c and d [5], respectively; their apoproteins are encoded by the nuclear genes *Lhcb4–6*, respectively [6] and so they are also referred to as the **Lhcb4–6** proteins (Lhcb1–3 are the LHCIIb apoproteins). The **Lhcb4** protein, or CP29, is the largest of these minor LHCIIb with an apparent molecular mass of 29 kDa; in the case of spinach, two or even three polypeptides are observed around 30 kDa upon denaturation [1]. It is not currently known if these polypeptides originate from different genes or are the products of post-translational modifications, although a phosphorylated form of Lhcb4 with a molecular mass of 34 kDa is observed in cold-treated maize leaves [7] (see below). All the minor LHCIIb are believed to form a structural and functional link between LHCIIb and the inner PSII antenna [8,9], and they have been predicted to play a role in the photoprotective dissipation of excess excitation energy, known as high-energy-state non-photochemical fluorescence quenching (q_E) [10,11]. Indeed, the carboxy-modifying agent dicyclohexylcarbodiimide has been found to bind to CP29 and CP26 (**Lhcb5** protein) in conditions where this reagent inhibits q_E [12]. At the same time these two complexes are enriched in the xanthophyll violaxanthin, a carotenoid believed to play an important role in energy

Correspondence to A. Pascal, SBPM/DBCM, CE-Saclay, F-91191

Gif-sur-Yvette, France. Fax: + 33 169084389, Tel.: + 33 169089015,

E-mail: apascal@cea.fr

Abbreviations: FWHM, full width at half maximum; LD, linear dichroism; LHC (I, II), light-harvesting complex (of photosystem I, II); PS (I, II), photosystem (I, II); q_E , high-energy state non-photochemical fluorescence quenching.

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dissipation [11,13], and the same two proteins show a higher level of q_E -related fluorescence quenching *in vitro* than other LHClIs [14]. Furthermore, phosphorylation-induced spectral changes in the **Lhcb4** protein during photoinhibitory cold treatments have been interpreted in terms of a possible involvement in down-regulation of light-harvesting [15]. Thus, as well their importance in light collection, CP29 and CP26 seem to play a central role in the balance between efficient light-harvesting and energy dissipation.

CP29 can be isolated from PSII membranes by non-denaturing IEF [16], cation-exchange chromatography [17] or Ca^{2+} -chelating chromatography [18]. While a large amount of spectral data has been gathered on the former preparation (see Results), the latter two have been the subject of less intense study as the isolation procedures did not allow full purification from other chlorophyll-binding proteins. Although the IEF procedure (followed by a further sucrose gradient step) generates the protein in a very pure form, it takes at least 2 days to isolate CP29 equivalent to a maximum of 0.5 mg chlorophyll (5–10% yield from PSII at 5 mg chlorophyll [16]). In addition, the running costs of flat-bed IEF, involving the use of ampholites and a gel composed of Ultradex (LKB), can be restrictive. Thus preparations based on the more routine, less expensive chromatographic techniques are of interest. Here we present a spectroscopic characterization of a preparation of the **Lhcb4** complex by a modified form of the protocol of Henrysson *et al.* [17] in which the protein has been purified to homogeneity. While complete purification takes up to a week, this can generate the equivalent of 4 mg chlorophyll of the protein while only consuming very basic laboratory reagents (cation-exchange resins are completely reusable). The spectral characteristics are in the main similar to those reported for the purified IEF fraction, but some significant differences remain. This conservation of spectral properties indicates that the data (for preparations involving two very different methods) are unlikely to represent artefacts introduced during purification and thus can safely be attributed to the native pigment–protein complex. The results are assessed in terms of the purity of this preparation; the attribution of absorption bands to specific pigments within the putative three-dimensional structure is also discussed.

MATERIALS AND METHODS

Isolation and purification of CP29

Spinach PSII membrane fragments were isolated as described previously [19]; extrinsic regulatory subunits of the water oxidase were released by incubation at $0.2 \text{ mg}\cdot\text{mL}^{-1}$ chlorophyll in 0.8 M Tris/HCl, pH 8.35, under room illumination for 30 min. The **Lhcb4** pigment-binding protein was purified from Tris-washed PSII using a modification of the protocol previously described by Henrysson *et al.* [17]. Sulfobetaine 12 (SB12; Serva) was used in place of sulfobetaine 14 (SB14) and the column equilibration buffer contained 0.1% (w/v) SB12 and 0.05% (w/v) *n*-dodecyl- β , D -maltoside (Boehringer-Mannheim) which greatly stabilized the pigment–protein complex. Chromatography was performed on a medium-pressure liquid chromatography system (Bio-Rad) under green light; a second chromatography step on a TSK CM column (TosoHaas) was necessary to remove contamination from the inner PSII antenna protein, CP47, and thus obtain sufficiently purified CP29. The protein was stored at -20°C in the presence of 2 mM benzamidine.

SDS/urea PAGE and immunological analysis

Polypeptide composition was analysed by SDS/urea PAGE using a 6% polyacrylamide stacking gel over a 15% separating gel (w/v), in the presence of 5 M urea and 0.1% (w/v) SDS; a discontinuous buffer system was used [20]. Gels were silver stained as described by Heukeshoven & Dernick [21].

Electroblotting was performed essentially as described in [22] except that poly(vinylidene difluoride) membranes (pore size $0.4 \mu\text{m}$) were used in place of nitrocellulose. Ascites fluid containing monoclonal antibodies (FC 8) directed against CP29 were used for immunological screening of the preparations (diluted 1 : 2000 in NaCl/P_i, pH 7.2, + 1% Tween-20; Sigma). Goat anti-mouse IgGs in the same buffer (1 : 2000 dilution) were applied as secondary antibodies in combination with 3-amino-9-ethylcarbazol (Sigma) as colouring reagent; 10 mg of the dye was dissolved in 5 mL dimethyl sulfoxide and added to 50 mL of 50 mM sodium acetate, pH 5.5, in the presence of 0.003% (v/v) H_2O_2 . The rainbow marker kit (46–2.5 kDa; Amersham) was co-transferred to the membranes to control the relative transfer efficiency and for molecular-mass determination.

Pigment analysis

Pigment composition was determined by reverse-phase HPLC as previously described [23]. The absorption coefficients used were those reported by Lichtenthaler [24] and Davies [25].

Spectroscopy

For low-temperature absorption, fluorescence and CD measurements, the protein was solubilized in buffer containing 0.06% (w/v) *n*-dodecyl- β - D -maltoside, 80% (v/v) glycerol and 20 mM Hepes, pH 7.5. Samples were cooled in a liquid nitrogen cryostat, a CF1204 helium-flow cryostat (Oxford Instruments) or a helium-bath cryostat (Utreks). Absorption and fluorescence spectra were recorded using $1 \times 1 \text{ cm}$ acrylic cuvettes, whereas for CD measurements a home-made plexiglass cuvette with a pathlength of 0.5 cm was used. Orientation of CP29 for linear dichroism (LD) measurements was achieved by a gel-compression technique [26]. Samples were dissolved in buffer (0.06% (w/v) *n*-dodecyl- β - D -maltoside, 60% (v/v) glycerol, 15 mM Hepes, pH 7.5) containing 15% (w/v) acrylamide/0.5% (w/v) *N,N'*-methylbisacrylamide, and the sample was polymerized at 4°C with 0.05% (w/v) ammonium persulphate + 0.03% (w/v) *N,N,N',N'*-tetramethylethylenediamine (Sigma) in a sample holder of $1.25 \times 1.25 \text{ cm}$ cross-section. Gels were compressed in two perpendicular directions (to $1 \text{ cm} \times 1 \text{ cm}$), allowing expansion along the third axis. After LD measurements, 77 K absorption spectra were recorded for the sample in the gel to confirm that polymerization had not resulted in deterioration of the protein.

Absorption spectra were measured on a Cary 219 spectrophotometer with a bandwidth of 1 nm. LD and CD were performed using a home-built spectropolarimeter with bandwidths of 1 nm and 3 nm, respectively. Fluorescence measurements were recorded with a charge-coupled device camera via a 1/2 m spectrograph (Chromex Chromcam I and 500IS); excitation light was provided by a tungsten halogen lamp fitted with a bandpass filter. Excitation was at 600 nm (bandwidth 20 nm) and the detection bandwidth was 0.5 nm.

RESULTS

Sample purity

Figure 1a shows an immunoblot challenged with monoclonal anti-CP29 and Fig. 1b a silver-stained SDS/urea polyacrylamide gel of the pigment–protein complex obtained after rechromatography on a TSK CM column. As can be seen from the figure the protein has been purified to substantial homogeneity, presenting the two bands around 30 kDa normally associated with CP29 in spinach [1,27], although there remained a very minor contribution from one or two lower-molecular-mass proteins (Fig. 1b) which did not react with the antibody (Fig. 1a). The very faint band around 21 kDa could be either CP24 or the PsbP protein (although this should not be present in Tris-washed PSII); it is unlikely to be the 22-kDa PsbS protein as this cross-reacts slightly with the antibody used (data not shown). The band around 27 kDa may correspond to CP26; note that while the antibody used in this study has previously been reported to cross-react with this protein, no such cross-reaction was observed in our hands for PSII particles (data not shown). Irrespective of the identity of these additional bands, the impurity is sufficiently minor (estimated at 1–2% total protein) as not to interfere with the spectroscopic measurements.

Pigment composition

Results of the pigment analysis are presented in Table 1. Based on a total of eight chlorophyllorophylls per Lhcb4 apoprotein [28], each CP29 protein would constitute 5.9 chlorophyll *a*, 2.1 chlorophyll *b*, 0.6 lutein, 0.6 neoxanthin and 0.7 violaxanthin. The chlorophyll *a* to chlorophyll *b* ratio (2.85) is practically identical to that of CP29 from *Zea mays* [15,28], and the proportion of xanthophylls is very similar (chlorophyll/carotenoid = 4.4, compared with 4.0 [29]); no measurable amounts of zeaxanthin or β -carotene were present.

The most significant difference in pigment stoichiometry between the CP29 preparation studied here and that obtained by non-denaturing IEF is seen in terms of the relative ratios of individual carotenoids per protein. Thus while approximately two xanthophylls per Lhcb4 have also been reported elsewhere [29] (the value of 3 previously reported for this preparation has been corrected in this paper), the amount of lutein found here is lower (0.67 compared with 0.85), while the stoichiometry of neoxanthin is greater, 0.6 here as compared with 0.5 based on the results of Giuffra *et al.* [29]. It is clear from the

Table 1. Pigment composition of CP29 determined by reverse-phase HPLC [23]. The absorption coefficients of Lichtenthaler [24] and Davies [25] were used. Values represent the mean \pm SD from five experiments and are expressed as molar ratios for Chl *a/b* (chlorophyll *a* : *b* ratio) and Chl/carot (chlorophyll : carotenoid ratio), or as percentages of total carotenoid for individual xanthophylls. Also presented, for comparison, are values published by Giuffra *et al.* [29]; note that these data have been converted to the same form.

| | This report | Giuffra <i>et al.</i> |
|----------------|-----------------|-----------------------|
| Chl <i>a/b</i> | 2.85 \pm 0.04 | 2.80 |
| Chl/carot | 4.4 \pm 0.3 | 4.04 |
| Lutein | 33.7 \pm 1.0 | 41.3 |
| Neoxanthin | 30.3 \pm 1.0 | 24.3 |
| Violaxanthin | 36.0 \pm 1.0 | 34.4 |

substoichiometric values found in each case that CP29 exhibits a flexibility in carotenoid binding and that any two of the three xanthophylls can insert into the two binding sites in the complex. It is assumed that these sites are analogous to the two carotenoid positions identified in the LHCIIB structure [3]. Giuffra *et al.* [28] also noted a variation in neoxanthin to violaxanthin ratio in reconstituted CP29, which was suggested to arise as a result of a competition for binding sites (no variation was reported in lutein stoichiometry). Note that in abscisic acid-deficient *Arabidopsis* mutants, where epoxy-xanthophylls (such as neoxanthin and violaxanthin) are not synthesized, it has been found that zeaxanthin replaces stoichiometrically the missing carotenoids within pigment–protein complexes (including LHCs) without either impairing assembly of these proteins or having any other apparent physiological consequences (e.g. [30]). In lettuce leaves the unusual carotenoid lactucoxanthin was found to replace up to two-thirds of bound lutein in LHC complexes when compared with similar fractions from other species [31]. It is also of interest to note, in this regard, the observed flexibility of CP29 with respect to chlorophyll *a/b* ratio; varying the pigment mixture allows reconstitution of proteins binding chlorophylls in differing ratios [28]. It is probable that this flexibility with respect to pigment-binding stoichiometry represents an adaptation to varying pigment supply, resulting from growth conditions, for example.

Absorption

The absorption spectrum of the CP29 preparation at 13 K is given in Fig. 2a, along with its second derivative. It displays bands corresponding to chlorophyll *a* in the Q_y (peak at 674 nm; weak shoulder around 664 nm) and Soret (438 nm peak) regions. Chlorophyll *b* exhibits two peaks in the Q_y region (638 and 650 nm), while in the Soret region it contributes to the peak around 464 nm. In the same area of the spectrum carotenoid absorption is present with distinct peaks at 496 and 483 nm. Similar peak positions have been observed for LHCIIB xanthophylls [23], although a minor carotenoid peak around 510 nm in LHCIIB is not evidenced here.

The temperature dependence of absorption in the Q_y region is presented in Fig. 2b (620–720 nm). At room temperature only a broad chlorophyll *b* absorption is observed, peaking at 640 nm, although a faint shoulder near 650 nm can just be discerned. Chlorophyll *a* absorption narrows upon cooling and in addition a shift in peak position is observed from 676.5 to 674 nm. Similar spectral features in this region have already been reported for CP29 [32,33]. The spectra are clearly different from those of the other PSII LHC proteins CP24, CP26 and LHCIIB [33]. Croce

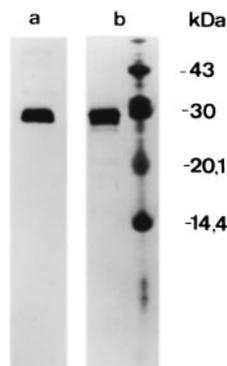


Fig. 1. Immunoblot (a) of purified CP29 after SDS/urea PAGE (b). In (b) SDS/urea PAGE of purified CP29 (left) was run against Pharmacia marker proteins (right); protein bands were visualized by silver staining.

et al. [15] reported small changes in the Q_y region upon phosphorylation, involving an increase in absorbance around 650 nm. The spectra presented here resemble those of unphosphorylated CP29 at low temperature (ratio between 638 and 650 nm chlorophyll *b* bands), while they appear more similar to phosphorylated CP29 at 290 K (around 660 nm). However, the 650 nm band as seen in Fig. 2 is better resolved than, for instance, in [29], where spectra at 71 K were presented for native and reconstituted CP29 with similar pigment content. In this previous report, two Gaussian bands were needed to describe the absorption around 650 nm. In contrast, only one band is required here; note also that the second-derivative spectrum (Fig. 2a) provides no evidence for a second band in this region.

In Fig. 2c the temperature-dependence of absorbance is shown for the Soret region (400–540 nm). The sharpening and shifting of carotenoid bands may possibly reflect a change in conformation upon cooling, although it is not uncommon

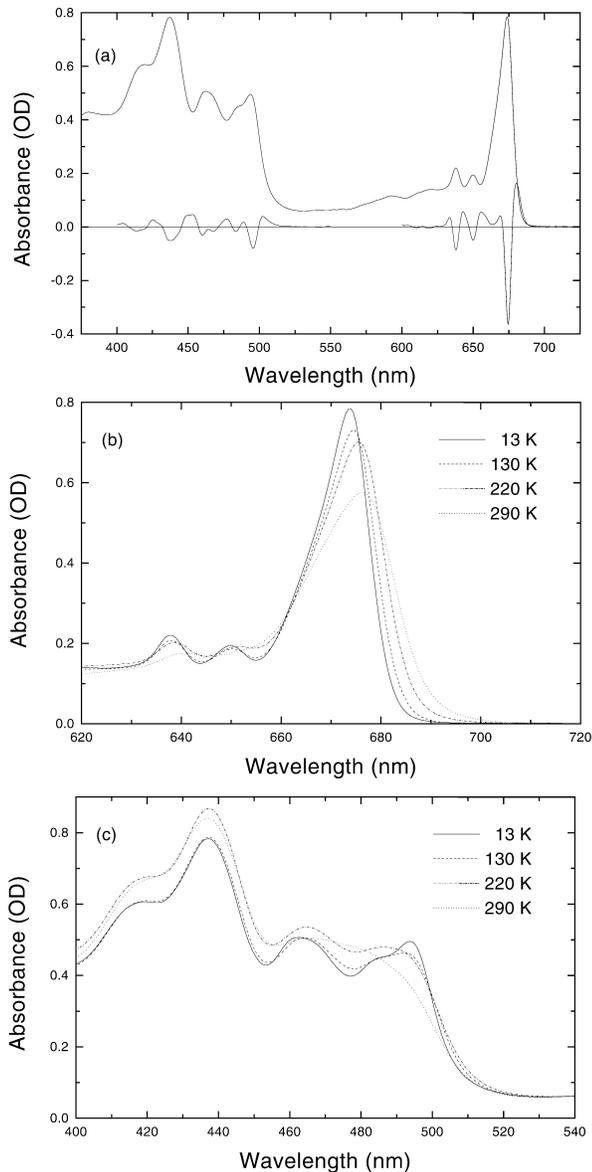


Fig. 2. Absorption spectra of CP29 (a) at 13 K (with second derivative) and (b, c) between 13 and 290 K. Spectra were recorded on a Cary 219 spectrophotometer with an optical bandwidth of 1 nm.

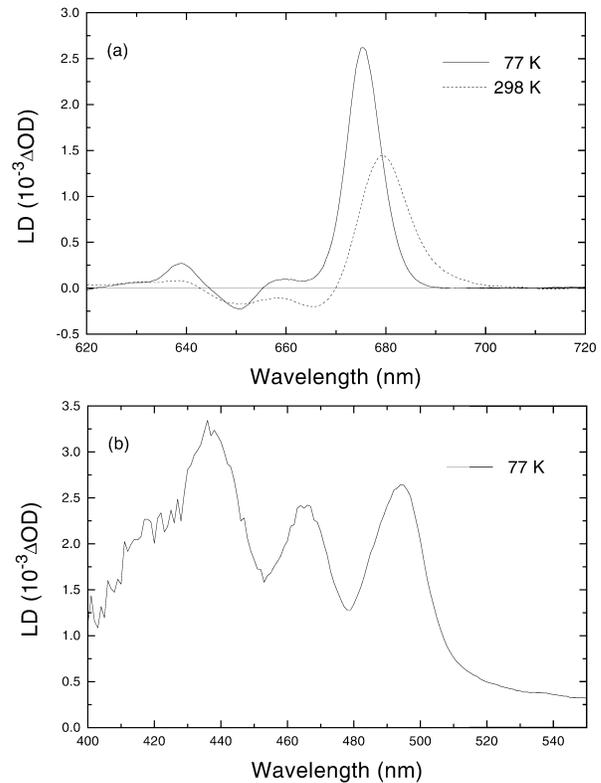


Fig. 3. LD spectra of CP29 at 298 and 77 K, recorded on a home-built spectropolarimeter with an optical bandwidth of 1 nm.

for carotenoids to show unusual temperature dependencies (e.g. [34]).

Linear dichroism

LD spectra of CP29 at room temperature and 77 K are shown in Fig. 3a between 620 and 720 nm. The spectra have the same overall appearance as those reported previously at 298 K and 10 K, respectively [33]. They are characterized by a large positive signal in the chlorophyll *a* region (675 nm at 77 K) and an oscillatory pattern in the chlorophyll *b* region. These appear to be common features for all chlorophyll *a/b*-binding proteins [33]. A blue-shift in the chlorophyll *a* region is observed from 679 to 675 nm upon cooling to 77 K, in line with the shift in absorption mentioned above. The room-temperature spectrum is somewhat different from that reported previously [33], being slightly negative around 660–665 nm in this case, but at low temperature the spectra are almost identical. The spectrum at 77 K resembles phosphorylated rather than unphosphorylated CP29, as reported by Croce *et al.* [15], in its shape around 660 nm. The positive and negative peaks at 639 and 650 nm clearly correspond to the chlorophyll *b* absorption bands at 638 and 650 nm, respectively. Note that almost identical chlorophyll *b* LD bands have been observed for the **Lhcb5** protein, CP26, although differences are seen around 660 nm [33,35].

The 77-K LD spectrum of CP29 in the Soret region is given in Fig. 3b (400–550 nm). The positive band at 494 nm corresponds to the carotenoid absorbing at 496 nm; xanthophylls also contribute to positive peaks around 465 and 437 nm, where the main absorption bands of chlorophylls *a* and *b* (respectively) are present. Note the large difference from the corresponding spectra of CP26 [35] and LHCIIB [26].

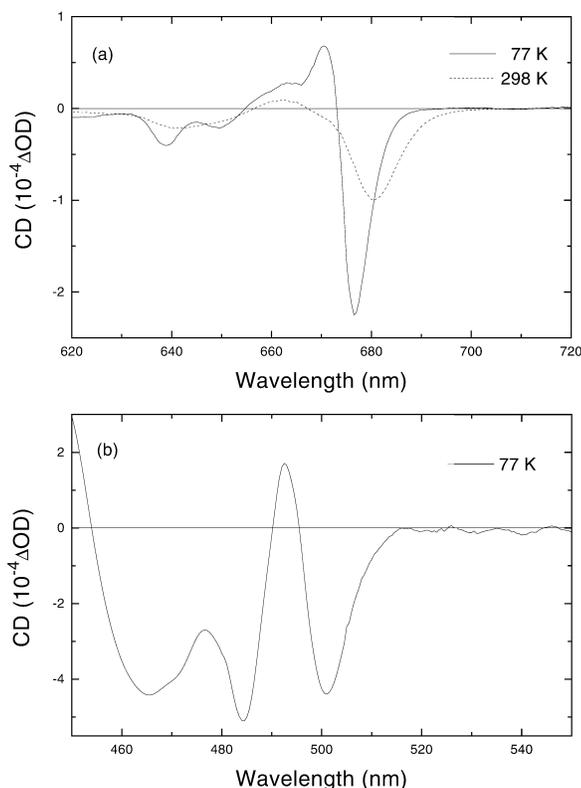


Fig. 4. CD spectra of CP29 at 298 and 77 K recorded with an optical bandwidth of 3 nm.

Circular dichroism

CD spectra at 298 and 77 K are shown in Fig. 4a for the Q_y region (620–720 nm). At 649 nm the CD intensity is clearly less than that at 639 nm (this is more clearly seen at 77 K than at room temperature), resembling the spectra reported for unphosphorylated CP29 from *Zea mays* [15] (note that this relationship was the other way around for the phosphorylated protein). The 77-K spectrum exhibits a positive band near 670 nm with a shoulder around 663 nm. Negative bands at 639 and 649 nm correlate with chlorophyll *b* absorption and LD bands at similar positions. Overall the CD in the chlorophyll *a* region is very similar to that of CP26 [35], with a strongly non-conservative

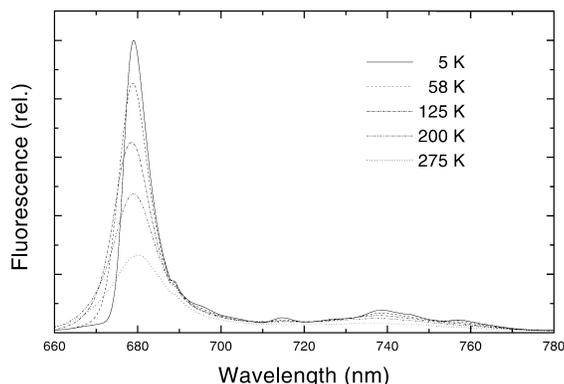


Fig. 5. Fluorescence emission spectra of CP29 between 5 and 275 K. Excitation was at 600 nm with an optical bandwidth of 20 nm. Spectra were recorded on a Chromex charge-coupled device camera with an optical bandwidth of 0.5 nm.

shape. It is also reminiscent of the CD of LHCIIb, although the latter is much more conservative [26]. CD in the chlorophyll *b* region is far less intense than for LHCIIb but again resembles that of CP26 a great deal [35]. Unlike LHCIIb (which contains more chlorophyll *b* molecules), the two minor PSII antenna proteins CP26 and CP29 have very similar chlorophyll *a* to chlorophyll *b* ratios. Together with the similar absorption and LD features in this wavelength region, this points to an almost identical organization of the two chlorophyll *b* molecules in both pigment–protein complexes. Although there exist some small differences, the overall spectral features in the Q_y region are very similar for the two proteins.

In the Soret region, CD spectra of CP29 at 77 K (Fig. 4b) differ to a large extent from those of CP26 [35]. This most probably reflects differences in carotenoid contributions in this region. The ratio of carotenoid bands around 465 and 484 nm (with the latter being the more negative) is similar to phosphorylated CP29 [15] (465 nm was reported to be the more negative for unphosphorylated CP29).

Fluorescence

Fluorescence emission spectra of CP29 at 5, 58, 125, 200 and 275 K are presented in Fig. 5. At 5 K, the emission peaks at 679.1 nm and has a bandwidth of 7 nm (FWHM). Upon increasing the temperature to around 100 K, the spectrum broadens and shifts to the blue. Above 125 K the spectrum continues to broaden but shifts to the red; at 275 K the emission peak is at 680.3 nm with a width of 14.6 nm (FWHM). This peculiar temperature-dependence of the fluorescence emission maximum was also observed for LHCIIb [26] and CP26 [35]. The room-temperature fluorescence emission spectrum for CP29 from *Zea mays* reported by Jennings *et al.* [36] differs slightly from that given here, with a peak position of 681.5 nm and a bandwidth of 16.7 nm (FWHM).

DISCUSSION

Although the sequence of CP29 has not yet been fully determined for spinach, the very high degree of homology across species for those sequences published (e.g. [1]) allows certain assumptions to be made about the possible binding sites of the chlorophyll molecules, based on the assignments in the atomic model of LHCIIb [3]. Microsequencing of small fragments of the spinach Lhcb4 polypeptide supports the validity of such assumptions (R. G. Walters, personal communication). Such a comparison of putative binding sites is summarized in Table 2 for CP29 and the related PSII LHC complex CP26. The preparation contains, based on a total of eight chlorophylls per protein [28], six bound chlorophyll *a* molecules and two chlorophyll *b* molecules. The exact positions of chlorophyll-binding sites in CP29 and the other minor LHCII proteins have been discussed at some length in the literature (e.g. [15,35,37,38]). More recent experiments on mutated Lhcb4 polypeptides have shed some light on these discrepancies (R. Bassi, personal communication). Thus the two chlorophyll *b*-binding sites correspond to positions b_3 and b_6 in the atomic model of LHCIIb, while the six chlorophylls *a* are bound at positions a_1 – a_5 and, surprisingly, the assumed chlorophyll *b* site b_5 . This latter site will be referred to as a_5' .

The two bound chlorophyll *b* molecules in CP29 can be correlated with the two Q_y absorption bands seen at low temperature (638 and 650 nm at 13 K; Fig. 2a). These bands also correspond to bands in LD (positive and negative peaks at 639 and 650 nm, respectively; Fig. 3a) and CD (negative peaks

Table 2. Comparison of identified chlorophyll-binding sites in LHCIb [3] with equivalent residues in CP26 and CP29 (from [1]). Note that for the latter two proteins, the cleavage site of the transit sequence is unknown and so the numbering may differ by one or two residues from other quoted sequences. Residues shown in italics are those changed from the sites in LHCIb, which may not therefore be chlorophyll-binding. For the three glutamate sites (a_1 , a_4 and b_5) the charge-compensating arginine is noted in parentheses; for a_6 , the proline unable to form a hydrogen-bond with the glycine peptide-carbonyl (or valine able to do so for CP29) is similarly indicated. For more details, see text.

| | LHCIb | CP29 | CP26 |
|----------------------|---------------|--------------------|----------------|
| Chl a_1 | E180 (R70) | E213 (R116) | E198 (R91) |
| Chl a_2 | N183 | <i>H216</i> | N201 |
| Chl a_3 | Q197 | Q230 | Q215 |
| Chl a_4 | E65 (R185) | E111 | E86 (R203) |
| Chl a_5 | H68 | H114 | H89 |
| Chl a_6 | G78 C=O (P82) | <i>G124 (V128)</i> | G99 C=O (P103) |
| Chl a_7 | ? | ? | ? |
| Chl b_1 | ? | ? | ? |
| Chl b_2 | ? | ? | ? |
| Chl b_3 | H212 | H245 | H230 |
| Chl b_5 (a_5') | E139 (R142) | E174 (R177) | E159 (R162) |
| Chl b_6 | Q131 | <i>E166</i> | <i>E151</i> |

at 639 and 650 nm; Fig. 4a) spectra at 77 K. Note that similar spectral features in the chlorophyll b Q_y region have been observed for CP26 [35]. A recent investigation of the energy-transfer processes in CP29 upon selective excitation of these two chlorophyll b Q_y transitions led to an assignment of each band to a particular position in the structure (i.e. chlorophyll b_3 responsible for 638 nm absorption and chlorophyll b_5 for the 650 nm band [38]). Reanalysis of these data in the light of the known positions of the chlorophyll b molecules (see above; data not presented) indicates that the only possible permutation still has chlorophyll b_3 absorbing at 638 nm, transferring excitation (~ 0.3 ps) to chlorophyll a_3 absorbing at 676 nm, while chlorophyll b_6 (650 nm) transfers to chlorophyll a_5' (670 nm) with a slower time constant (~ 2 ps). Note that the previous calculation on these results had not allowed for the possibility of a chlorophyll a molecule at position ' b_5 '.

In the chlorophyll a Q_y region, absorption spectra of CP29 exhibit a principal band around 675 nm (674 nm at 13 K; Fig. 2a) which, as for LHCIb [26] and CP26 [35], has a composite character. The 675-nm LD peak at 77 K (absorption peak at 674 nm at 77 K) with a sharp decrease towards 670 nm (Fig. 3a), along with a positive CD band at this wavelength (Fig. 4a), indicates the presence of an absorption band at 670 nm (as for LHCIb and CP26). Note that an absorption band near this wavelength has already been suggested for all PSII LHC proteins [32,33]. Given the overall similarity in spectral properties of the principal 675 nm band for these LHC proteins (strong positive LD, strong negative CD), it would most probably correspond to chlorophylls a having similar positions in the structure of all LHCI complexes. Part of the red absorption band shifts to the blue upon cooling (Fig. 2b), again similar to the behaviour of CP26 and LHCIb. The presence at room temperature of a weak band around 684 nm which disappears upon cooling has been concluded for all LHCIIs by Gaussian decomposition of absorption spectra [33], which would be in agreement with a blue shift in the reddest band. CP29 also exhibits a weak shoulder in absorption spectra, around 664 nm at 13 K (Fig. 2a). This band may correspond to a broad positive CD feature around 663 nm (Fig. 4a), and to a

positive CD feature in LHCIb and CP26. The principal difference between LHCIb and CP26 in the chlorophyll a region is the distinct 670-nm absorption in the bulk complex [35]. Like CP26, CP29 shows evidence of only a weak absorption band in this region.

The apparent absence of the presumed chlorophyll a -binding sites a_6 and a_7 in CP29 (see above) may indicate correlation of (at least some of) chlorophylls a_1 – a_5 and a_5' with the common absorption band (in LHCIb, CP29 and CP26) around 675 nm, and of chlorophyll a_6 and/or a_7 (thus not present in CP29) with a part of the 670 nm absorption in LHCIb (note that the asparagine-ligating chlorophyll a_2 in LHCIb is replaced with a histidine in CP29, as is the case for CP24 [1]). The glycine residue proposed to provide the ligating peptide-carbonyl to chlorophyll a_6 (Gly78 in LHCIb [3]) is present in CP29 and CP26, whereas the proline (position 82 in LHCIb) is present in CP26 but is replaced by valine in CP29 (and CP24 [1]). As this residue is (unlike proline) able to hydrogen-bond to the glycine peptide-carbonyl, chlorophyll ligation is not possible at this site in CP29 (see Table 2).

Comparing the data presented here with those of the IEF preparation, it is clear that the basic spectral properties are identical. Given the differences in isolation techniques, this is a good indication that these properties are intrinsic to the native membrane protein and have not been modified during either purification. Considering the small changes in spectral properties reported by Croce *et al.* [15], brought about by cold stress-induced phosphorylation, the spectra presented here show some similarities to both phosphorylated and unphosphorylated CP29 from maize. This preparation does contain at least one phosphorylated threonine residue, but this is not reversible by treatment with alkaline phosphatase (data not shown) and is likely to be a permanent, post-translational modification to the polypeptide unrelated to cold stress. The polypeptide profile of the current preparation, having bands around 29–30 kDa (Fig. 1) rather than exhibiting the dramatic shift to 34 kDa associated with the stress phenomenon, is consistent with this interpretation. It is not clear whether this represents a species difference between CP29 from maize and spinach, or whether native CP29 from non-cold-treated maize exhibits the same phosphorylation pattern. We are currently investigating whether further stress-related phosphorylation of the protein has similar effects to those observed for the complex isolated from maize.

This protocol generates higher yields over equivalent time scales than that of Dainese *et al.* [16], 4–5 days giving up to 4 mg chlorophyll for this preparation compared with 2 days yielding approximately 0.5 mg chlorophyll by IEF, while being less demanding in terms of specialized equipment and cost. It should therefore be of great utility in experiments (in particular spectroscopic ones) requiring large quantities of sample, the recent pump-probe measurements carried out on this preparation being a prime example [38].

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