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The intrinsic 22 kDa protein is a chlorophyll-binding subunit of photosystem II

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Abstract

The intrinsic 22 kDa polypeptide associated with photosystem II (psbS protein) was found to be able to bind chlorophyll. Extraction of isolated photosystem II membranes with octyl-thioglucopyranoside, followed by repetitive electrophoresis under partially denaturing conditions gave only one green band. It contained both chlorophyll *a* and chlorophyll *b*, exhibited an absorption maximum at 674 nm and a 77 K fluorescence peak at 675 nm. The chlorophyll–protein band contained a single polypeptide of 22 kDa. Based on these results and on previous protein sequence comparisons, it is suggested that the psbS protein is a chlorophyll *a/b* binding polypeptide and should thus be denoted CP22.

Key words: Chlorophyll-protein; psbS gene product; 22 kDa protein; Photosystem II; Spinach

1. Introduction

A 22 kDa protein was earlier found to be co-precipitated from detergent-solubilized PSII membranes using antibodies raised against the extrinsic 33 and 23 kDa proteins associated with the photosynthetic water-splitting system [1]. Based upon these studies it was suggested that this protein was an integral subunit of the PSII complex. This suggestion was experimentally substantiated by the presence of the 22 kDa protein in isolated PSII core preparations [2,3] and by its location in the grana partition regions of the plant thylakoid membrane [4,5]. The protein can be isolated by ion exchange chromatography after detergent solubilization of PSII enriched membrane particles [4,6]. The purified protein showed a very hydrophobic character, but did not contain any cofactors. In particular, the isolated protein did not bind chlorophyll or any other pigments.

The function of the 22 kDa protein is still unknown. It has been inferred to have a role in stabilising the acceptor side of PSII [3] and to confer DCMU sensitivity [7] or to have a structural role for the binding of extrinsic PSII proteins to the inner thylakoid surface [4]. However, more recent experiments argue against these earlier suggestions [8,9].

The 22 kDa protein is nuclear encoded (psbS gene) and synthesised as a precursor of 274 amino acids with an N-terminal transit peptide of 69 amino acids [10,11]. The mature protein is predicted to possess four transmembrane helices. Quite unexpectedly, the sequence of the psbS protein revealed a striking resemblance to the various chlorophyll *a/b* antenna proteins [10,11] and a more limited relatedness to the ELIPs [12]. In the present study we demonstrate that the 22 kDa protein from spinach can bind both chlorophyll *a* and chlorophyll *b* and therefore constitutes a previously undiscovered pigment-binding protein associated with PSII.

2. Materials and methods

PSII membrane fragments (BBY) were isolated from spinach leaves as in [13]. A fraction enriched in the 22 kDa polypeptide was obtained by incubating the PSII membranes, suspended in 0.4 M sucrose, 25 mM MES-NaOH, pH 6.0 and 15 mM NaCl at 0.5 mg Chl · ml^{-1}, with 0.6% octyl-thioglucopyranoside (OTG) for 5 min on ice in the dark as in [8]. The suspension was centrifuged at 40,000 × *g* for 30 min and the greenish pellet, referred to as the OTG-pellet [8], was resuspended in 40% glycerol and used either directly or stored at −30°C. For successful isolation of this fraction, it was important that starch, DNA and other

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Abbreviations: BBY, photosystem II membrane fragments; CAB, chlorophyll *a/b* binding; Chl, chlorophyll; CP, chlorophyll-binding protein; ELIP, early light induced protein; MES, 4-morpholine-ethanesulphonic acid; OGP, octyl-glucopyranoside; OTG, octyl-thio-glucopyranoside; PSII, photosystem II; PVDF, polyvinylidenefluoride; SDS-PAGE: SDS-polyacrylamide gel electrophoresis.

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high molecular mass contaminants were removed by a low speed centrifugation (10,000 × g for 4 min) prior to sedimenting the BBY membrane fragments at 40,000 × g [14].

Samples for partially denaturing SDS-PAGE were solubilized with octyl-glucopyranoside (OGP) at a detergent/chlorophyll ratio of 30:1 and loaded on a 10% polyacrylamide gel made with the gel buffer described in [15]. After electrophoresis at 0°C for five to seven hours in the dark, the resulting green bands were excised from the gel and loaded directly onto a second gel made with 8% polyacrylamide [16]. For polypeptide analysis, the gel pieces were denatured with 2% SDS and separated by fully denaturing SDS-PAGE according to [17] using 17.5% acrylamide and 4 M urea. The gels were stained with Coomassie brilliant blue R-250 or electroblotted onto a PVDF membrane for immunostaining. Antibodies raised against purified 22 kDa protein [4], LHCII, CP24 [18], CP29 [19], CP26 [20] and CP14 [21] were used for immuno-decoration with ^{125}I-labelled protein A for detection [22]. Chlorophyll proteins were electroeluted from partially denaturing SDS-PAGE and the protein concentration was determined according [23] with modifications described in [24]. The chlorophyll content was determined in 80% acetone as in [25] and recalculated according to [26].

Absorption spectra were recorded with a Shimadzu UV-3000 double-beam spectrophotometer. To analyse the chlorophyll–protein complexes resolved by mild SDS-PAGE, the gels were placed in a cuvette with 40% glycerol and scanned at 650 and 675 nm [27]. Fluorescence measurements of the green bands, cut from the gel, were recorded at 77 K with a Perkin Elmer luminescence spectrometer. For emission spectra the samples were excited at 435 nm; for the excitation spectra the emission was measured at 675 nm.

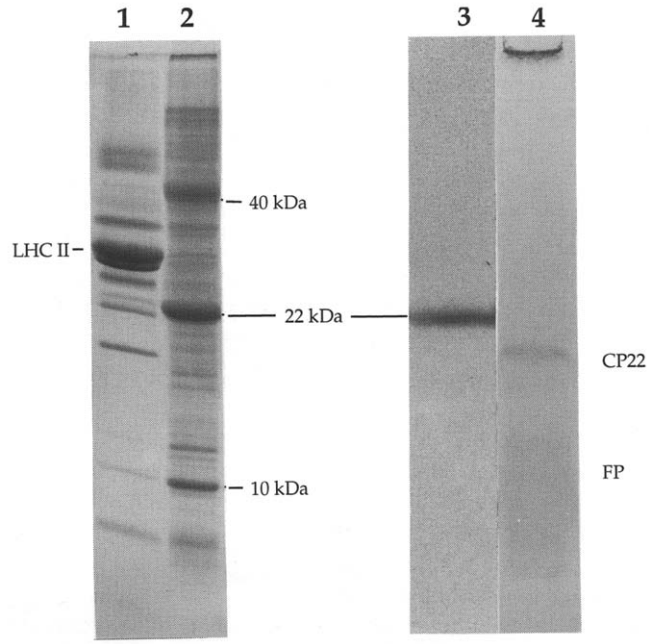

Fig. 1. Denaturing, Coomassie-stained SDS-PAGE to show the polypeptide components of PSII membrane fragments (lane 1) and the OTG-pellet (lane 2). Partially denaturing SDS-PAGE (10% acrylamide) of the OTG-pellet (lane 4). The green band was cut from the gel, run on denaturing SDS-PAGE and then immunodecorated with antibodies raised against the 22 kDa protein (lane 3). FP, free pigment.

3. Results

The 'OTG-pellet' was isolated from BBY membrane fragments by extraction with octyl-thioglucanopyranoside and centrifugation [8]. On denaturing SDS-PAGE (Fig. 1, lane 2) there are three predominant polypeptides of 22 kDa, 40 and 10 kDa. Other polypeptides are present only in minor amounts. When antibodies raised against the purified psbS protein [4] were used, only the 22 kDa protein was immunodecorated (not shown). Thus the OTG extraction procedure gives a pronounced enrichment of the 22 kDa protein from the BBY membrane fragments while the LHCII is very much depleted (cf. Fig. 1, lane 1). The ratio of the 22 kDa protein to

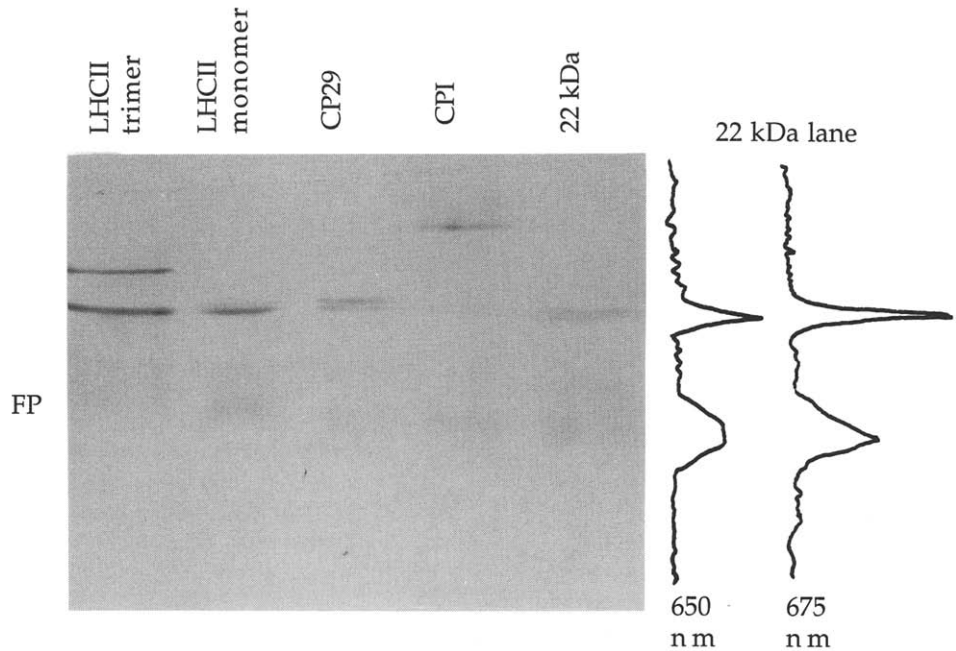

Fig. 2. Mild SDS-PAGE (8% acrylamide) of bands cut from a partially denaturing SDS-PAGE (10% acrylamide). LHCII trimer, LHCII monomer, CP29 and CPI were cut from a thylakoid sample and the green 22 kDa slice from the OTG-pellet. FP, free pigment. Right side: absorption of the 22 kDa band gel slices scanned at 650 nm and 675 nm.

LHCII is as high as 136 in the OTG-pellet as revealed from scans of Coomassie stained gels. The 10 kDa protein was shown by immunostaining to correspond to the psbR gene product [4,28] in accordance with [8] while N-terminal sequencing revealed the 40 kDa polypeptide as ferredoxin-NADP reductase (not shown).

In the next experiment, the OTG-pellet was solubilized with octyl-glucopyranoside and electrophoresed on a gentle SDS-PAGE under conditions which preserve chlorophyll binding to proteins. As seen in Fig. 1, lane 4, only one major green band could be seen to migrate into the gel. The absence of a visible green band corresponding to LHCII confirmed its low abundance in the OTG-pellet. When the green band from the OTG-pellet was excised and rerun on a fully denaturing gel, a single polypeptide in the 22 kDa region was obtained. This polypeptide was immunodetected by the antibody raised against the purified psbS protein (Fig. 1, lane 3).

Some chlorophyll containing material aggregated in the stacking gel and did not enter the separation gel. This material contained mainly the 22 kDa polypeptide as well as some LHCII, 10 kDa and 40 kDa polypeptides (not shown). This aggregation is due to the very hydrophobic nature of the OTG-pellet making complete solubilization difficult to achieve. If increased concentrations of detergent were used all the chlorophyll became detached and migrated as free chlorophyll during electrophoresis.

To exclude the possibility that the chlorophyll apparently associated with the 22 kDa polypeptide might be due to its comigration with other chlorophyll-binding proteins during the mild SDS-PAGE, the green band obtained from the 10% polyacrylamide gel (Fig. 1, lane 4) was excised and subjected to a second partially denaturing electrophoresis on an 8% polyacrylamide gel. Again, a single green band was obtained (Fig. 2, last lane). The electrophoretic migration of this green band was compared to that of other re-electrophoresed chlorophyll–protein bands obtained from mild SDS-PAGE of thylakoid membranes. It migrated somewhat faster than the CP29 band and was well separated from CPI and free chlorophyll. However, it migrated at about the same position as the monomeric form of LHCII. This observation probably explains why this green band can not be directly resolved during mild SDS-PAGE of thylakoids or PSII enriched particles. Moreover, it should be kept in mind that the OTG-pellet represents a very minor proportion (less than 1%) of the total chlorophyll associated with PSII.

To further exclude the possibility that the chlorophyll-

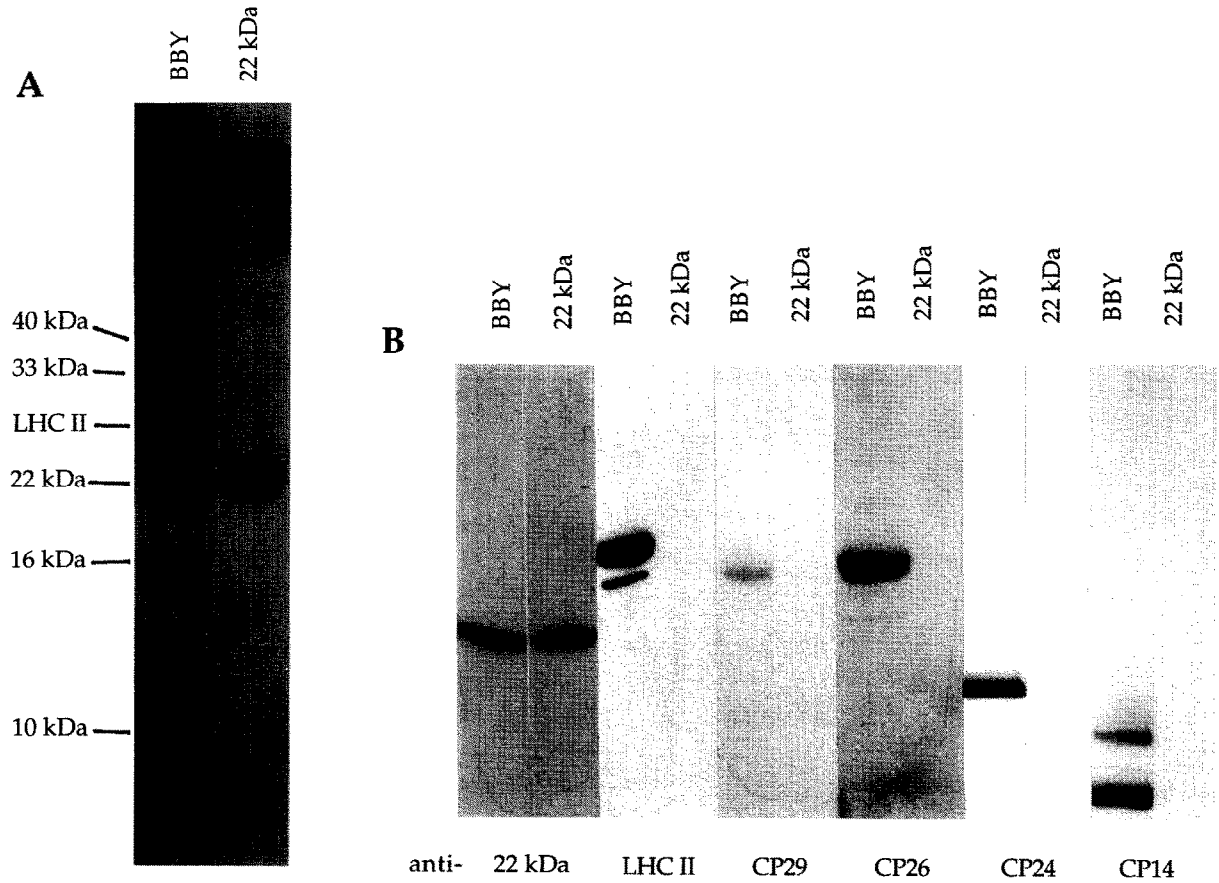

Fig. 3. Denaturing SDS-PAGE (17.5% acrylamide, 4M urea) and immunoblots of PSII membrane fragments and purified CP22. (A) Coomassie stained lanes. (B) Immunoblots of PSII membrane fragments (as positive control) and CP22 using antisera against the 22 kDa protein, LHCII, CP29, CP26, CP24 and CP14.

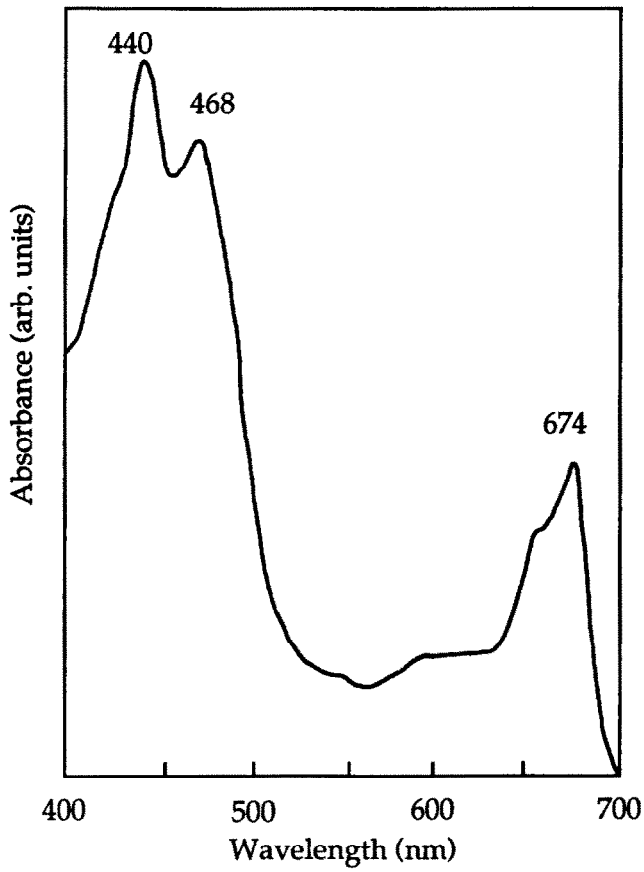

Fig. 4. Absorption spectrum of purified CP22 in polyacrylamide gel slice.

containing band from the OTG-pellet might be due to one of the known CAB gene products, the purified band from the second 'green gel' was denatured and electrophoresed on a totally denaturing gel (Fig. 3A). Coomassie staining showed it contained a single polypeptide of 22 kDa. Duplicate samples were electrotransferred to PVDF membranes and challenged with antibodies against LHCII, CP29, CP26, CP24, and a 14 kDa protein suggested to bind chlorophyll [21] (Fig. 3B). BBY membrane fragments were used as positive controls. All the antibodies reacted strongly with polypeptides in the BBY preparation, but none of them gave any cross-reactivity against the polypeptide of the purified green band except for the antibody raised against the psbS protein (Fig. 3B, left side). We therefore conclude that this green band represents a distinct chlorophyll–protein complex whose apo-polypeptide is the psbS gene product. It will consequently be referred to as 'CP22'.

The room temperature absorption spectrum of the green gel slice obtained after the second mild SDS-PAGE is shown in Fig. 4. An absorption peak can be seen at 674 nm in addition to two maxima (440 nm and 468 nm) in the blue region of the spectrum. The shoulder at 650 nm indicates the presence of chlorophyll *b*. The presence of chlorophyll *b* associated with the 22 kDa protein was further established by integration of the absorption scans (Fig. 2, right side) made of the green gels at 650 nm and 675 nm [27], which gave a chlorophyll *a*/*b* ratio of 1.6. Furthermore electroeluted 22 kDa protein was extracted with 80% acetone according to [25]. The chlorophyll content calculated according to [26] gave a chlorophyll *a*/*b* ratio of 2.2 compared to the starting OTG-pellet which showed a ratio of 4.3. The amount of carotenoids associated with the green band appeared to be quite low as judged from the absence of a distinct shoulder at 490 nm.

The fluorescence excitation spectrum of the green gel slice (Fig. 5A) showed strong peaks at 420 nm (chlorophyll *a*), 440 nm and 466 nm. This suggests that a pigment different from chlorophyll *a*, probably chlorophyll *b*, is transferring excitation energy to the chlorophyll *a* in the complex. The low temperature (77K) fluorescence emission spectrum (Fig. 5B) showed a main peak at

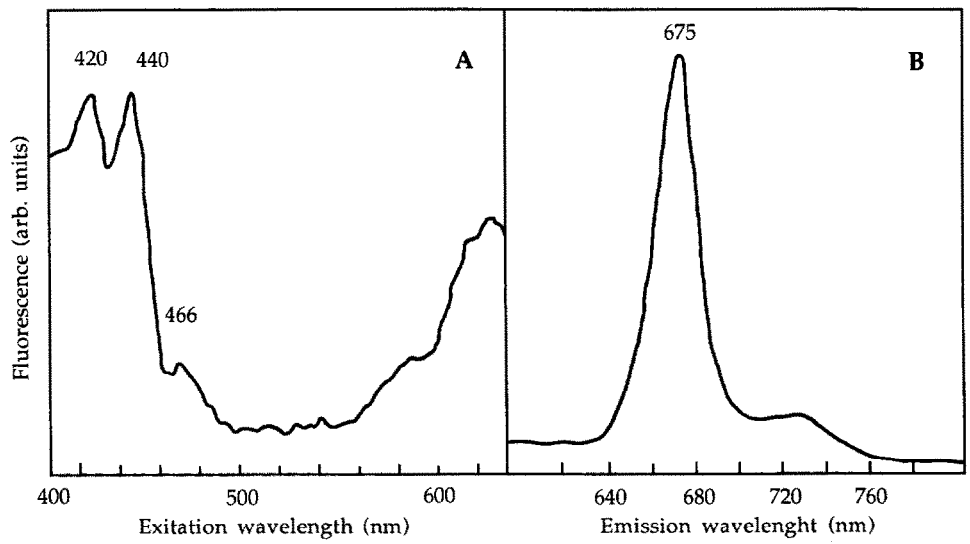

Fig. 5. Fluorescence spectrum of purified CP22 in polyacrylamide gel slice, recorded at 77 K. (A) Excitation spectrum with emission measured at 675 nm. (B) Emission spectrum obtained upon excitation at 435 nm.

675 nm. The small peak near 730 nm is attributed to the vibronic satellite.

The chlorophyll/protein molar ratio of purified CP22 obtained from the gel slice after the two concerted mild SDS-PAGE runs had a value of 3–4. LHCII subjected to the same treatment had a ratio of 10–11.

4. Discussion

The function of the intrinsic 22 kDa protein (psbS) is still unclear. Suggested functional and structural roles of this protein associated with either the acceptor or donor side of PSII have not been substantiated [3,8,9]. More recently, a possible role in chlorophyll binding has been inferred from sequencing studies which revealed a strong homology between the 22 kDa protein and the various CAB-gene products and the ELIPs [10,11]. In this study we have been able to verify experimentally that the 22 kDa protein of higher plants is able to bind chlorophyll. This was demonstrated through experiments involving selective extraction of BBY membrane fragments with the detergent OTG [8] combined with analysis using partially denaturing SDS-PAGE.

The following evidence demonstrate that chlorophyll is associated with the 22 kDa protein: (i) two consecutive electrophoretic runs using different acrylamide concentrations preserved a distinct green band, clearly separated from free chlorophyll; (ii) denaturing SDS-PAGE of the green band revealed only one major polypeptide (22 kDa) with minimal contamination by other polypeptides; (iii) this polypeptide was immunostained with antibodies raised against the psbS protein, but not with antibodies raised against other PSII CAB-proteins. The 22 kDa polypeptide binds both chlorophyll *a* and *b*. The 77 K fluorescence measurements indicate that the energy transfer is operational. This in turn implies a functional binding of chlorophyll to the 22 kDa protein and argues against binding of pigment due to non-specific hydrophobic association.

Analyses of the green gel slice after two electrophoretic runs revealed a chlorophyll *a/b* ratio of 1.8 and a chlorophyll/protein molar ratio of 3–4. However, considering the relatively high amount of free chlorophyll produced during the electrophoretic procedures these values may not be very applicable to the native chlorophyll–protein complex. In fact, the chlorophyll characteristics of the OTG-pellet probably represent a more accurate estimation of the 22 kDa protein considering its dominance over other CAB proteins in this fraction. The average chlorophyll *a/b* ratio of the OTG-pellet is 4.3, which corresponds to 3.1 according to the Arnon method. Such a ratio for 'CP22' would make it comparable to other low abundance chlorophyll *a/b* proteins of PSII such as CP29 (Chl *a/b* 2–4) [19] and CP26 (Chl *a/b* 2.2–2.7) [29,30]. Assuming that all the chlorophyll liberated during the electrophoretic runs is originating solely from the 22 kDa protein its chlorophyll/protein molar ratio would be 6.

The sequence of the psbS gene which encodes the 22 kDa protein, shows that it has four trans-membrane helices, rather than the three helices of the related CAB proteins and ELIPs. This suggested that their most recent common ancestor may have had four helices, with the C-terminal one subsequently lost in the CAB and ELIP lineages [10,11,31]. The modern 'CP22' may fulfil the role of a minor light-harvesting antenna in PSII. Alternatively, it may bind chlorophyll in a transient manner. It could, for example, be involved in transfer of newly synthesised chlorophyll to other antenna proteins during biogenesis, or might bind chlorophyll molecules liberated during degradation and turn-over of chlorophyll binding proteins. These latter functions would be in line with some of the roles suggested for the ELIPs [32].

It is interesting to note that this protein has been immunologically detected in the cyanobacterial species *Synechocystis* 6803 [33] and *Phormidium laminosum* (unpublished data), which only contains chlorophyll *a*. If a four-helix psbS protein-like ancestor gave rise to the extended family of three-helix chlorophyll *a/b* proteins [31], possibly the function of this extra helix was to stabilise the chlorophyll–protein in a manner to allow the complex to be stable with only chlorophyll *a*, or perhaps with chlorophyll biosynthesis precursors. It is clear that more developmental and evolutionary studies will be needed to elucidate the role of this protein in the photosynthetic membrane.

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