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Biochimica et Biophysica Acta, 1365 (1998) 207–214

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Higher plants light harvesting proteins. Structure and function as revealed by mutation analysis of either protein or chromophore moieties

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Received 27 January 1998; received in revised form 12 March 1998; accepted 12 March 1998

Abstract

Mutation analysis of higher plants light harvesting proteins has been prevented for a long time by the lack of a suitable expression system providing chromophores essential for the folding of these membrane-intrinsic pigment–protein complexes. Early work on in vitro reconstitution of the major light harvesting complex of photosystem II (LHCII) indicated an alternative way to mutation analysis of these proteins. A new procedure for in vitro refolding of the four light harvesting complexes of photosystem II, namely CP24, CP29, CP26 and LHCII yields recombinant pigment–proteins indistinguishable from the native proteins isolated from leaves. This method allows both the performing of single point mutations on protein sequence and the exchange of the chromophores bound to the protein scaffold. We review here recent results obtained by this method on the pigment-binding properties, on the chlorophyll-binding residues, on the identification of proton-binding sites and on the role of xanthophylls in the regulation of light harvesting function. © 1998 Elsevier Science B.V.

Keywords: Photosynthesis; Chlorophyll-binding residue; Carotenoid; Photoprotection

1. Introduction

1.1. A common structure for light harvesting complex (Lhc) proteins

Colours characterising Earth's landscapes are mostly due to chlorophyll and carotenoids bound to a class of proteins called *Lhc* which are inserted into the thylakoid membranes of plant chloroplasts and are homologous to each other. Their nature as hydrophobic, noncovalent, chlorophyll *a*-, chlorophyll *b*- and xanthophyll-binding proteins has been first recognised in the case of the major light harvesting complex of photosystem II (PSII; LHCII), the most abundant and stable member of this polypeptide family [1]. CP29 was then detected [2] followed by LHCI [3], CP24 [4] and CP26 [5] while other members of the *Lhc* family, like CP22 [6] and ELIPs (early light induced proteins) [7], have been recognised on the basis of their protein or cDNA deduced sequences, although their pigment-binding properties are still not clearly defined. The *Lhc* family includes now at least ten members in higher plants [8] while the finding that Chlorophytes, Rhodophytes, *Chromophytes* and *Dinoflagellates* have antenna pro-

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Abbreviations: DCCD, dicyclohexylcarbodiimide; LHCII, light harvesting complex of PSII; PS, photosystem.

PII: S0005-2728(98)00068-1

teins which also belong to *Lhc*, makes this protein family even larger and important for the elucidation of plant phylogeny (for a review see Ref. [9]). Review articles on antenna proteins have appeared [9–16]. The reader is addressed to these studies for comprehensive description of antenna systems. In this short review we report on the recent aspects of the structure and function of *Lhc* proteins in higher plants that have emerged from mutation analysis. Through the paper the nomenclature of Bassi et al. [17] will be used for chlorophyll–proteins while apoproteins will be addressed according to the consensus nomenclature of Jansson et al. [8]; the discussion is mainly restricted to PSII proteins (*Lhcb*) for reasons of space and also because most advancements have been accomplished within this group.

Our knowledge of *Lhc* protein structure arises from the resolution of higher plants LHCII complex at near-atomic resolution [18] and from the conclusion that this structure provides a general model for the overall folding of all the *Lhc* proteins since they have substantial regions of sequence conservation [18,19] noticeably in the membrane spanning domains as shown in Fig. 1. The pigment-binding characteristics of *Lhcb* gene products are summarised in Table 1.

In selecting a model system for the study of *Lhc* proteins, the data in Fig. 1 and Table 1 must be considered. The best object, in principle, would be LHCII since its structure has been experimentally determined and therefore can be used as a guideline for mutation analysis. Moreover, LHCII has been studied for over 30 years by many groups and its characterisation is advanced. However, a number of drawbacks make LHCII unsuitable, namely: (i) it is an heterogeneous protein made by the products of many highly homologous genes [20] thus making the comparison of a recombinant protein with its native counterpart impossible; (ii) it is a hetero-trimeric protein in which protein–protein interactions are as important as the intrasubunit features in determining the biochemical and spectroscopical characteristics of the system thus making it difficult to identify the

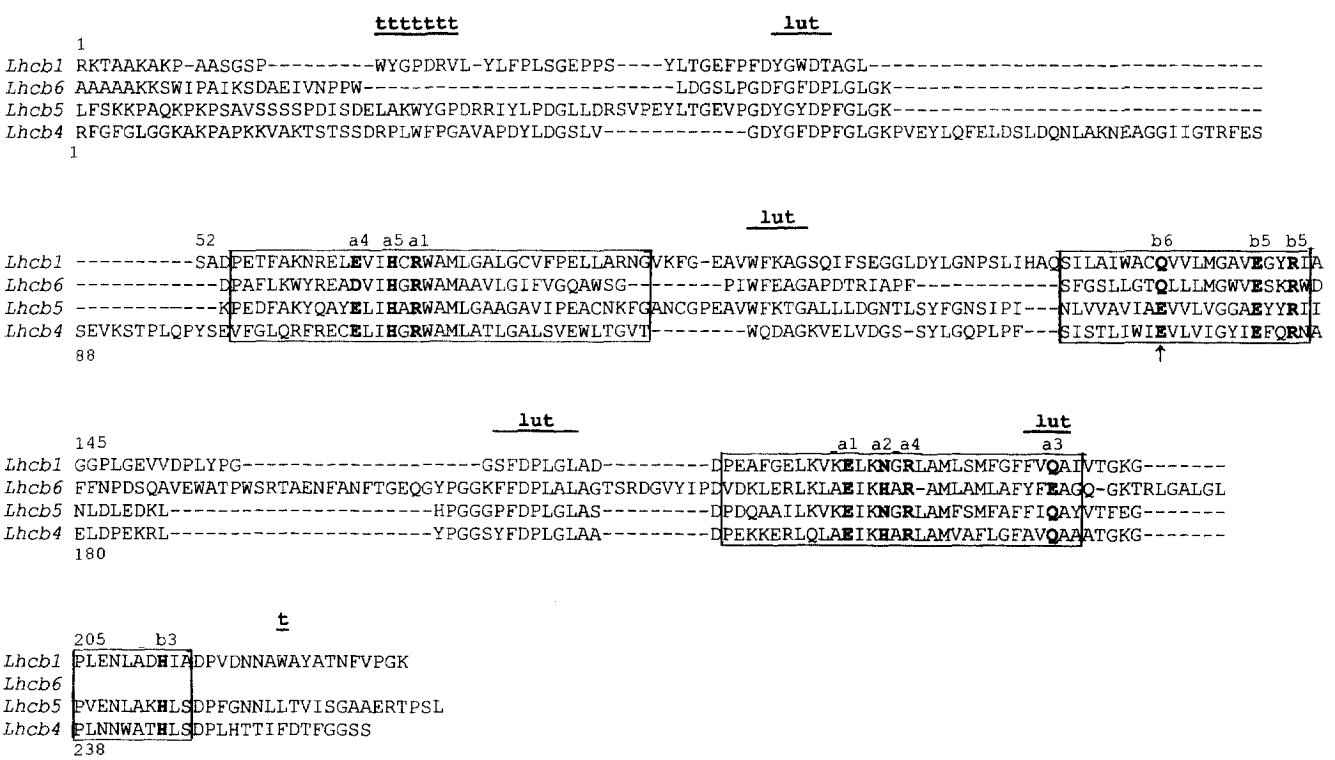

Fig. 1. Alignment of *Lhcb1*, *Lhcb4*, *Lhcb5* and *Lhcb6* cDNA derived sequences. Bold letters indicates residues identified as coordinating chlorophyll molecules in LHCII. Arrow indicates the DCCD binding site in CP29 (*Lhcb4*) (see text for further comments). lut, indicates consensus sequences for xanthophyll binding. ttttt, indicates sites involved in trimerisation of LHCII. Sequences boxed, membrane spanning domains (helices A–C) and the amphiphilic helix D.

Table 1
Biochemical properties of *Lhcb* proteins

	nLHCII	nCP29	nCP26	nCP24	rCP29	References
Chlorophyll *a*	7	6	6	5	6	[18,51]
Chlorophyll *b*	5	2	3	5	2	[23,30,31]
Conserved chlorophyll-binding residues respect to LHCII	8[a]	7	7	6	7	See Fig. 1
Xanthophylls	3/4	2	2	2	2	[21–23,52]
Unidentified chlorophyll-binding residues	4	1	2	3	1	See Fig. 1
Aggregation state	Trimer	Monomer	Monomer	Monomer	Monomer	[41,30]

[a] All of the chlorophyll-binding residues so far identified.
n, Native protein extracted from thylakoids; r, recombinant protein obtained by in vitro refolding of *E. coli* overexpressed apoprotein.

primary effect of point mutations; (iii) it has a variable carotenoid content depending on growth conditions and occupancy of two sites whose location in the protein has not been resolved by structural studies; (iv) four chlorophyll-binding ligands have not been identified making them unaccessible to mutation analysis. For the above reasons, we have instead chosen *Lhcb4* (CP29) since it is homogeneous, monomeric, has only two xanthophyll-binding sites and eight chlorophyll-binding sites for seven of which the ligand can be easily identified by homology with LHCII.

Lhc are integral membrane proteins with an helix–loop–helix organisation. A model based on Kühlbrandt et al.'s structure [18] and on sequence analysis of the *Lhcb4* (CP29) gene product is shown in Fig. 2. The apoprotein is 257 amino acids long and each polypeptide binds eight chlorophyll molecules (six chlorophyll *a* and two chlorophyll *b*), and two xanthophylls (lutein, violaxanthin and neoxanthin in nonstoichiometric amounts) [18,21–23]. Three transmembrane domains, with α-helix conformation, are connected by two hydrophilic loops on either sides of the membrane while N-terminal and C-terminal peptides are exposed respectively on the stromal and lumenal spaces bearing each a small helix domain. The N-terminal peptide is fully hydrophilic, thus protruding into the stromal space. Sequence comparison has identified four putative xanthophyll-binding sequences in *Lhc* proteins [15] one of which, GFDPF (residues 48–52), is located at the centre of the N-terminal hydrophilic domain, suggesting that the sequence in between E_{100} and G_{53} forms a loop while only the first 47 amino acids are free in the stroma. The threonine 83 (T_{83}) has been found to be reversibly phosphorylated as part of a CK2 (casein kinase 2) site [24,25] thus inducing a conformational change in the membrane intrinsic domain of the molecule [26]. The lumen exposed C-terminal sequence is fully hydrophilic in the D_{248} to S_{257} stretch which is thought to protrude in the lumen while the S_{247} to P_{237} sequence form an amphiphilic helix, lying on the membrane surface, as detected by electron crystallography in LHCII. The helix B is the nearest to the N-terminal; it is 51 Å long, starting at V_{101} and extending until T_{153} in 9.5 turns. Helix A is 43 Å long, extending from P_{203} to A_{232} in eight turns. These two domains are held together by interhelix ionic pairs formed respectively by the charged residues R_{116} and E_{213} and by E_{111} and R_{218} thus forming an X-shaped structure. Since the length of both helices and the cross-bridging residues are conserved, the axes of helices are likely to be tilted by 32° relative to the membrane normal plane as they are in LHCII. The two buried ion pairs would provide a strong attractive force between the two helices and are likely to play a major role in stabilising the protein in the membrane. The first 24 residues of helices A and B are homologous to each other and related by an axis of local twofold symmetry running perpendicular to the membrane plane. The helix C, shorter than A and B, runs from S_{158} to N_{178} in 5.5 turns, over a length of 31 Å, with a tilt angle of 9° relative to the membrane normal plane and is end-capped by an intrahelix ionic pair E_{174}–R_{177}. The helix D, from P_{238} to S_{247} is parallel to the membrane plane. The 3.4 Å resolution allows elucidation of the chromophore coordination in most cases. Out of the 12 chlorophyll molecules resolved by electron crystallography in LHCII, only eight are conserved in CP29. Chlorophyll *a* and chlorophyll *b* cannot be distinguished in the structure because of the small differences between them. Two xanthophyll molecules can be located at the centre of the complex on

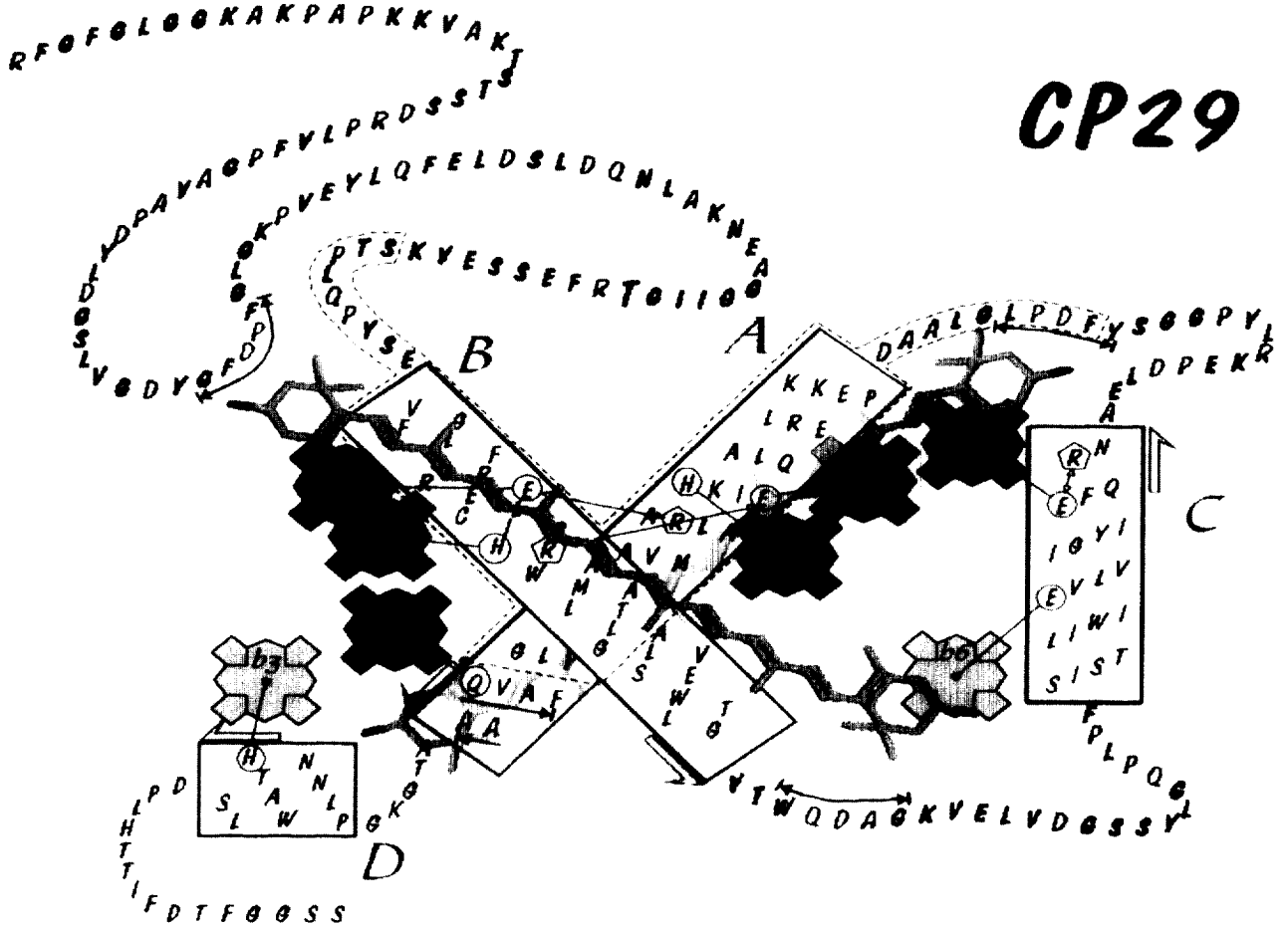

Fig. 2. Model of CP29 pigment–protein in the thylakoid membrane. Letters circled indicate chlorophyll side-chain ligands. Arginines, charge compensating glutamate side-chains, are encircled in a pentagon and connected with a line. Xanthophyll molecules are drawn in positions suggested by their location in the structure [18] and according to the consensus binding sequence identified [15] which is boxed with grey background. Dashed outlines indicate the regions of the polypeptide related by inner homology in the sequence.

either sides of helices A and B with an angle of 50° with the membrane normal plane. In CP29 two xanthophyll molecules are present per polypeptide [23] and yet lutein, neoxanthin and violaxanthin are consistently found thus implying the two sites can accommodate different carotenoids with similar structure. These form an internal cross brace in the centre of the complex, providing a direct, strong link between the peptide loops at both surfaces. The four sites appear to be a derivative of the consensus sequence WFDPL and are located close to both ends of membrane spanning domains A and B [15]. The porphyrin rings have been attributed to chlorophyll *a* or chlorophyll *b* on the basis of their proximity to the carotenoids [18]. The identification of porphyrin ligands can be made on the basis of homology with LHCII in six out of eight cases: two histidines (H_{216} and H_{245}), one amide Q_{230}, three charge compensated glutamates, forming ion pairs with arginines either in the same helix (E_{174}–R_{177}) or in another helix (R_{116}–E_{213} and E_{111}–R_{218}). Two residues identified as chlorophyll ligands in LHCII are not conserved in CP29: N_{183} is substituted by H_{114} and Q_{197} by E_{166}; while histidine is a suitable ligand for chlorophyll, glutamate can not coordinate a Mg-porphyrin unless it is charge compensated.

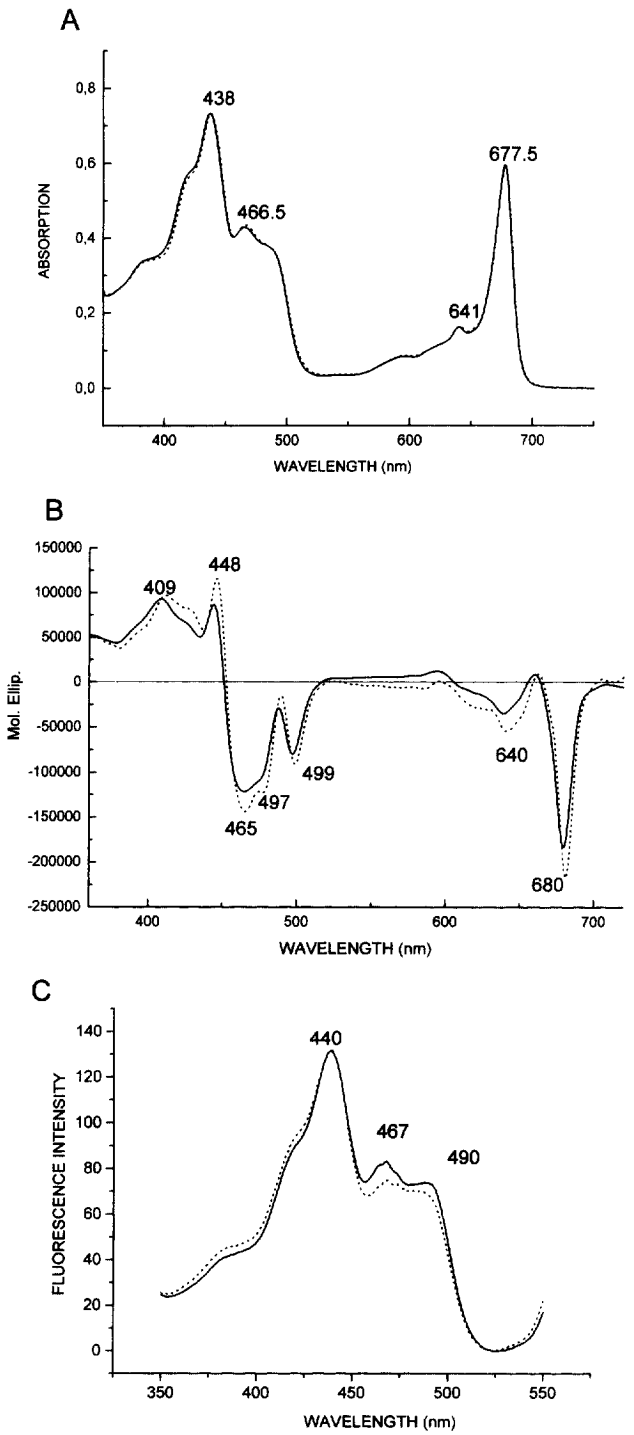

Fig. 3. Comparative analysis of native and recombinant CP29. (A) Absorption spectra; (B) circular dichroism; (C) fluorescence excitation spectra (for 681 nm emission). Solid line, native CP29; broken line, recombinant CP29.

2. In vitro refolding of CP29 pigment–protein from the *Lhcb4* gene product overexpressed in *E. coli* and purified chromophores

The task of devising an experimental refolding procedure by which up to 15 chromophores (chlorophyll *a*, chlorophyll *b*, lutein, neoxanthin, violaxanthin in different stoichiometries) can be noncovalently bound in a correct conformation to a 30 kDa hydrophobic apoprotein could be regarded as hopeless. However, pigment binding to LHCII apoproteins upon in vitro refolding had been previously described [27,28]. The characteristics of the reconstituted pigment–proteins were, however, rather different with respect to those of the native protein extracted from leaves [29] making them unsuitable for mutation studies. The definition of an improved procedure [30] yielded recombinant pigment–proteins indistinguishable from their native counterparts by using a variety of biochemical and spectroscopical techniques. Fig. 3A–C and Table 1 summarise the properties of native and recombinant CP29 supporting the view they are essentially identical. Similar results can be obtained with CP24 [31], CP26 [32] and LHCII (Varotto, Sandonà and Bassi, unpublished results) although in each case the folding conditions must be adapted to the specific protein. Fig. 4 shows the dependence of the ratio between chlorophyll *a* and chlorophyll *b* in the recombinant proteins on the relative concentration

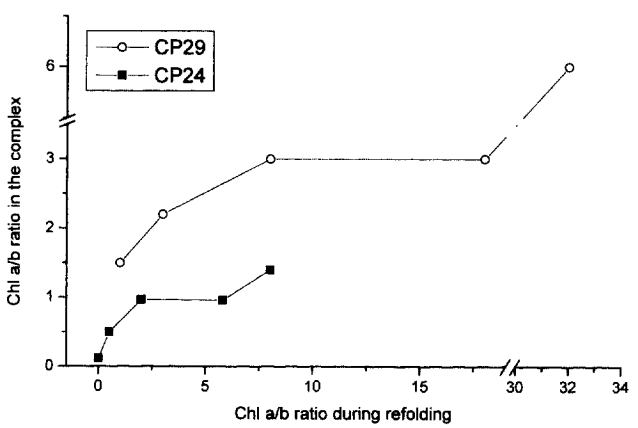

Fig. 4. Dependence of the ratio between chlorophyll *a* and chlorophyll *b* chromophores in the reconstituted protein on the ratio in the refolding solution. The plateau range is found at values corresponding to those of the native protein extracted from thylakoids.

of the chromophores in the refolding solution for CP29 and CP24. In each case a range is found in which the chlorophyll *a*/*b* ratio in the complex does not change with the chlorophyll *a*/*b* ratio in the folding environment. Since the plateau values correspond to those found in the native proteins, it is suggested that the native conformation corresponds to an high stability state for the proteins. In each case the total number of chlorophylls bound to recombinant proteins is the same thus implying low selectivity of the binding sites for chlorophyll *a* and chlorophyll *b* [23]. Similar dependence was found for the xanthophyll-binding sites.

3. Identification of chlorophyll *a* and chlorophyll *b* absorptions in *Lhc* proteins

The function of *Lhc* proteins is absorption of light energy, the transfer of excitons to reaction centres and the harmless dissipation of excess energy. In this respect the absorption characteristics of chromophores is highly significant for protein function. While only two chemically distinct chlorophyll species are present in antenna complexes, many optical transitions (spectral forms) are commonly observed in the Qy absorption region [29,33,34]. Lack of progress in understanding this spectroscopic heterogeneity has been mainly due to the absence of experimental techniques making possible selective modification of the optical transitions. Also, it has not been possible to assign a particular transition to chlorophyll *a* or chlorophyll *b* though it is generally assumed that the shorter wavelength bands are associated with chlorophyll *b*. Analysis of a recombinant CP24 refolded in the presence of excess chlorophyll *b* allowed identification of four absorption forms peaking at 638, 645, 652 and 659 nm whose amplitude in the absorption spectrum was greatly increased. On the other hand four chlorophyll *a* absorption forms (666 nm, 673 nm, 679 nm and 686 nm) were decreased in amplitude or absent in the high chlorophyll *b* complex [23,31]. Similar analysis of recombinant CP29 with altered chlorophyll *a* vs. chlorophyll *b* binding showed that pigment–protein rather than pigment–pigment interactions determine the tuning of chromophore absorption wavelengths in chlorophyll *a*/*b* proteins [23].

4. Regulation of light harvesting function by protonation of lumen-exposed residues and violaxanthin to zeaxanthin conversion

Fluorescence quenching in the photosynthetic apparatus of higher plants is originated by a set of physiological mechanisms which channel excess excitation energy away from reaction centres when light intensity exceeds CO_2 availability or the transport capacity of the electron transfer chain. This is triggered by the intensity of pH gradient [35,36] and modulated by deepoxidation of the xanthophyll violaxanthin to zeaxanthin [37,38]. CP29 and CP26 have high violaxanthin and zeaxanthin content [21,38] and bind the quenching inhibitor dicychlohexylcarbodiimide (DCCD) [39,40] a protein modifying agent binding to proton sensitive residues. Analysis of recombinant CP29 with WT sequence or carrying the single point mutation $E_{166}Q$ showed that the mutation inhibits DCCD binding [22]. The effect of xanthophyll deepoxidation has been studied by spectroscopical analysis of recombinant CP29 containing either violaxanthin or zeaxanthin. It was shown that the latter complex showed 30% lower fluorescence yield and enhanced amplitude of a short lifetime component (1.7 ns) (Crimi, Bassi and Holzwarth, unpublished results). These results support the view that CP29 is one of the sites of the qE (energy quenching) quenching mechanism in PSII. Additional quenching sites can be CP26 and CP24 as suggested by their high zeaxanthin content following induction of NPQ [26,38].

5. Identification of chromophores and their transition energy levels in CP29 structure

Energy transfer in chlorophyll-binding proteins is regulated by three major parameters: (i) distance between chromophores; (ii) mutual orientation of transition dipoles; (iii) energy levels of absorption and fluorescence. While structural studies on LHCII have elucidated the first point and the second could in principle be obtained, with improving resolution of the protein, by locating the phytol chains, the determination of absorption energy levels cannot be determined by structural studies. We have approached this problem by constructing a series of point mutants

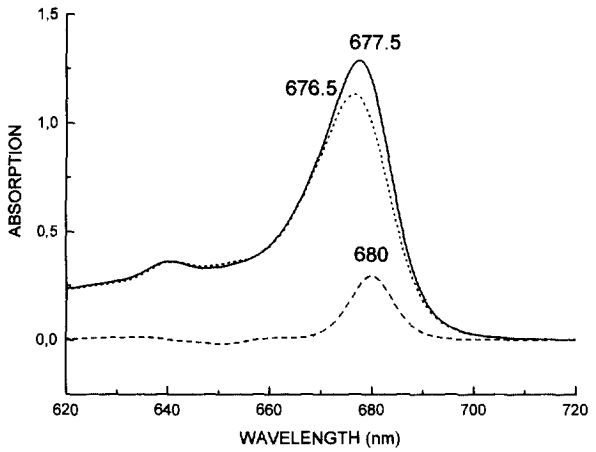

Fig. 5. Absorption spectra of the WTCP29 (solid), of the $H_{216}F$ mutant (dotted) and WT−$H_{216}F$ difference spectrum (broken). The difference spectrum is thought to represent the absorption spectrum of a single chlorophyll *a* molecule in CP29 thus identifying at 680 nm its absorption energy level.

on the putative chlorophyll-binding residues. Seven mutants were shown to loose a single chlorophyll and yet bound seven out of eight chlorophyll *a*+*b* molecules per polypeptide. In one case mutation of the putative chlorophyll-binding residue led to complete disassembly of the protein. By differential absorption spectroscopy, the absorption energy levels of seven out of eight chlorophylls in CP29 has been determined (Sandonà, Cugini, Croce and Bassi, unpublished results). Fig. 5 shows the WTCP29 minus $H_{216}F$ mutant absorption spectrum as an example of absorption energy determination of a single chlorophyll in an antenna complex.

6. Subunit interactions in the antenna system: trimerisation of LHCII

When thylakoid membranes are solubilised, *Lhc* proteins can be brought to detergent solution, and in these conditions the major LHCII complex is trimeric [41] although it may monomerise upon phosphorylation [42,43]. CP24, CP29 and CP26 are monomeric in detergent solution although the former two can form an heterooligomeric complex with LHCII [43]. It clearly appears that the understanding of protein–protein interaction determinants in the thylakoid membrane is of fundamental importance in studying photosystem organisation and physiology. Up to now a systematic study has only been possible in the case of LHCII trimerisation since LHCII has been reconstituted in vitro into monomers [27,28] and trimers [44] thus allowing mutational analysis. Determinants of LHCII trimerisation are present in both N-terminal and C-terminal domains (Fig. 1). Chymotryptic removal of N-terminal peptide including a lipid-binding site induces monomerisation [45]. The protein segment comprising amino acids 16–21 has been identified as a trimerisation motif [46] and is thought to be involved in the binding of a lipid containing *trans*-hexadecanoic acid which is indispensable for trimerisation in vivo [47]. In the C-terminal domain, ten amino acid residues can be removed without affecting trimerisation while deletion or substitution of the tryptophan residue in position 222 abolishes trimerisation [48] both in vitro and after insertion in thylakoid membrane thus destabilising the monomeric protein which is rapidly degraded [49]. The extension of this approach to other *Lhc* proteins will be important in understanding photosystem assembly. In this context the recent report of reconstitution of *Lhca1* and *Lhca4* gene products into heterodimers [50] offers new opportunities.

Acknowledgements

Roberto Simonetto is thanked for help in the preparation of figures. Work on chlorophyll–proteins was supported by the "Piano Nazionale Biotecnologie Vegetali" from the Italian Ministry of Agriculture and Forestry to R.B.

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