Chapter 10

LHCI: The Antenna Complex of Photosystem I in Plants and Green Algae

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Summary

In this chapter we summarize the results of reports published since 1979 on the Chl *a*/*b*-binding light-harvesting complex of PS I. In the first part of this chapter, we review the results that led to our current knowledge of the biochemical properties of the individual gene products constituting LHCI, in particular, the presence and distribution of red-shifted spectral forms and the idea that LHCI, different from LHCII, is organized into heterodimeric complexes. Recent developments based on EM and X-ray crystallography have been reported, which led to the understanding that LHCI is bound to only one side of the PS I–LHCI supramolecular complex rather than surrounding the reaction center as was recently found in bacterial photosystems. The evidence that each Lhca subunit is present in a single copy per reaction center is also reported as compared with the data leading to a previous model with two copies of Lhca polypeptides. In the second part of this chapter, we focus on the origin of the peculiar characteristic of LHCI: the red-shifted Chl spectral forms. First, the evidence for their origin from Chl–Chl interactions is reviewed; second, the different models for the organization of the chromophores involved in these interactions, which have been recently proposed, are discussed. We conclude that present evidence favors the origin of red forms from Chl *a*–Chl *a* excitonic interaction between chromophores localized in binding sites A5 and possibly B5 of the Lhca proteins, while the evidence for involvement of additional chromophores such as Chl *b* are consistent with an indirect structural role of the ligand in site B6.

I. Introduction: LHCI Within the PS I Supercomplex

Photosystem I (PS I) is a multisubunit complex located in the stroma lamellae of thylakoid membranes, which functions as light-dependent plastocyanin/ferredoxin oxidoreductase. In higher plants and green algae this supramolecular complex is composed by two chlorophyll (Chl) binding moieties: (i) the core complex and (ii) the external antenna, encoded by either chloroplast or nuclear genes, respectively. The core is composed by 14 subunits, among which PsaA and PsaB bind the primary donor P700, and cofactors of the electron transport chain up to F_X as well as the greatest portion of the 96–103 Chl *a* and 22 β-carotene molecules. In fact, only a few Chls are bound to the minor subunits of the core complex (Jordan et al., 2001; Scheller et al., 2001; Ben Shem et al., 2003; see also Fromme and Grotjohann, this volume, Chapter 6; Nelson and Ben-Shem, this volume, Chapter 7). In addition to the inner antenna Chls bound to the core moiety, plant PS I is equipped with a peripheral antenna system in which Chl *a*, Chl *b*, and carotenoid molecules are bound to four polypeptides, namely Lhca1–4, with molecular weights between 21 and 24 kDa (Haworth et al., 1983; Bassi and Simpson, 1987; Jansson, 1994). The polypeptide composition of PS I–LHCI, PS I-core, and LHCI is shown in Fig. 1.

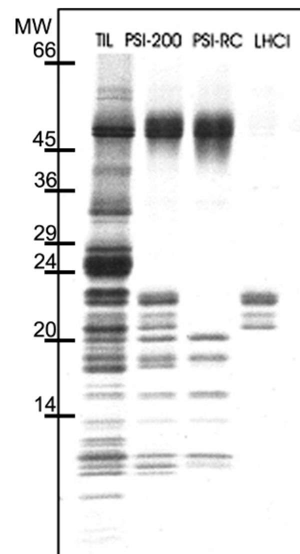

Fig. 1. Polypeptide composition of the PS I–LHCI supercomplex and its two moieties: the PS I-core complex binding Chl *a* and β-carotene and the LHCI moiety binding also Chl *b* and the xanthophylls lutein and violaxanthin. The separation was performed by SDS–urea PAGE. Source: Croce et al. (2002).

The topological organization of the LHCI moiety within the PS I supercomplex remained obscure until recent electron microscope (EM) studies by single particle analysis (Boekema et al., 2001b) showed that the Lhca subunits are located asymmetrically, on one side only, of a monomeric core complex. This organization is in contrast to that of PS II, in which a dimeric core

Abbreviations: α (β)-DM – *n*-dodecyl-α(β)-D-maltoside; β-car – β-carotene; Car – carotenoid; CD – circular dichroism; Chl – chlorophyll; EM – electron microscope; FWHM – full width half maximum; LD – linear dichroism; Lhc – light-harvesting complex; OGP – *n*-octyl-β-D-glucopyranoside; PS I(II) – photosystem I(II); Viola – violaxanthin; WT – wild type.

Fig. 2. Localization of LHCI polypeptides within the PS I–LHCI complex resolved by X-ray diffraction. Top (stromal) view of the PS I–LHCI complex is shown. The four Lhca subunits are aligned into a crescent-like structure on the lower part of the figure. Subunits exclusive of eukaryotic PS I complex are also indicated. Figure was reproduced from Ben Shem et al. (2003) with permission of the authors.

complex is symmetrically surrounded by peripheral antenna proteins that bind at multiple sites (Boekema et al., 1999). Nearest-neighbor analysis was performed both by covalent cross-linking and by observing the loss of Lhca components upon knockout or antisense depletion of individual core complex subunits. On this basis it was proposed that Lhca3 may interact with PsaK, Lhca2 with PsaG, and Lhca1/Lhca4 with PsaF and PsaJ (Jansson et al., 1996; Jensen et al., 2000; Scheller et al., 2001). Moreover, it was also suggested that each Lhca component is in contact with two others (Jansson et al., 1996).

An alternative model has been recently proposed based on the 4.4 Å crystal structure of PS I–LHCI complex from pea (Ben Shem et al., 2003). Lhca complexes were localized asymmetrically on one side of the core complex moiety, aligned in a crescent-like structure (Fig. 2). The only clear interaction could be observed between the helix C of Lhca1 and PsaG, while Lhca2, Lhca3, and Lhca4 subunits are proposed to interact with the core components trough small binding surfaces at their stromal-exposed regions. Both structural and biochemical data support the view of a LHCI antenna moiety arranged on one side only of the PS I-core complex. This arrangement is rather unusual among photosynthetic systems that are generally characterized by a circular or symmetrical arrangement of antenna subunits around the reaction center complex (Bibby et al., 2001; Boekema et al., 2001a; Roszak et al., 2003). The reason for this topological organization appears to be a structural adaptation to allow State I–State II transitions (Allen, 1992). This mechanism provides a balance in energy transfer to PS II versus PS I by transfer of phosphorylated LHCII subunits to stroma lamellae, where they serve to increase the antenna size for PS I. The docking of LHCII to the PS I-core complex has been reported to be mediated by the PsaH subunit (Lunde et al., 2000), which is located on the LHCI-free side of the PS I-core and is thus accessible for binding the phosphorylated LHCII.

From a spectroscopic point of view, the most streaking feature of PS I is the presence of Chls that absorb at

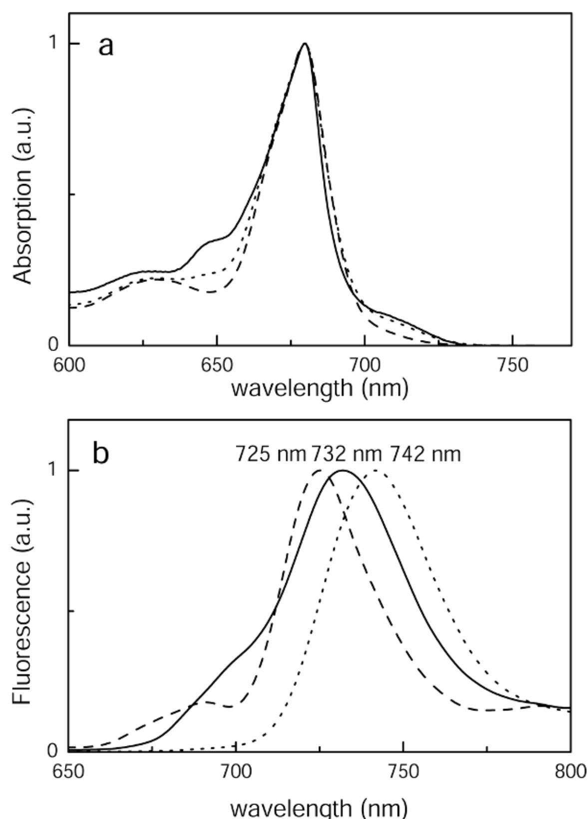

Fig. 3. Spectroscopic properties of LHCI (solid), PS I-core (dashes), PS I–LHCI (dots). (a) Absorption spectra at 77 K (b) Fluorescence spectra at 77 K, emission maxima are also reported.

energy lower than P700, the reaction center trap (Butler and Norris, 1963). This is clearly visible in the fluorescence emission spectra of the PS I–LHCI complex peaking at approximately 740 nm. The absorption and emission spectra of PS I–LHCI, PS I-core, and LHCI are reported in Fig. 3. The absorption spectra show that the Chl *b*, which is responsible for the absorption at 475 and 650 nm, is associated with LHCI proteins, as are the absorption forms at wavelengths >700 nm, which form the red-most tail in the spectra.

The fluorescence emission spectra of the PS I–LHCI supercomplex and of its two moieties reflect the distribution of red-most absorption forms. Thus, PS I–LHCI, PS I-core, and LHCI exhibit their fluorescence emission peaks at 740, 720, and 735 nm, respectively (Mullet et al., 1980b).

It has been demonstrated that at physiological temperatures, 80–90% of the excited states in the system reside in the red-shifted forms (Croce et al., 1996). This implies that to be used for charge separation, most of the excitation energy in PS I has to be transferred uphill from red absorption forms to P700, the reaction center trap.

II. Characterization of LHCI

A. Identification of Lhca Genes with Their Gene Products

Lhca proteins are encoded by the nuclear genome, synthesized by cytoplasmic ribosomes, imported across the two membranes of the chloroplast envelope, and finally inserted into the thylakoid membrane (Lubben et al., 1988). Lhca gene sequences are now available from different organisms, thanks to the work of sequencing projects (e.g., see TIGR web site, http://www.tigr.org/tdb/tgi/plant.shtml).

In vascular plants, six classes of Lhca genes have been identified, namely *Lhca1–6*. Moreover, several copies of the same gene can be found, depending on the species (see, e.g., Jansson, 1994 and references therein). In Fig. 4A, the alignment of protein sequences of Lhca from *Arabidopsis thaliana* is shown (Jansson, 1994). Biochemical studies on Arabidopsis as well as on other plants have shown that only the proteins encoded by genes *Lhca1–4* are expressed under the different experimental conditions investigated (see section II.B and references therein). No information is at present available for the products of the genes *Lhca5* and *Lhca6* except that they are transcribed at a very low level (Jansson, 1999). However, the possibility that Lhca5 and Lhca6 are expressed under special environmental conditions or developmental states cannot be ruled out. Lhca6, which is highly homologous to Lhca2, was proposed to be a pseudogene (Jansson, 1999).

The Lhca genes belong to the Lhc multigenic family, which also includes the genes for the outer antenna of PS II, namely *Lhcb1–6* (Jansson, 1999). In Fig. 4B an unrooted cladogram created by ClustalX of all Lhc deduced protein sequences from Arabidopsis is shown. Antenna proteins of PS I cluster together, being more homologous to each other than to the Lhcb PS II components. Interesting exceptions from this general trend are Lhca1, which is more related to Lhcb4 that to Lhca2–4, and Lhcb6, which is intermediate between the two clusters. Evolutionary studies on the Lhc multigenic family confirmed a separation between Lhca and Lhcb proteins; in fact, Durnford et al. (1999) proposed that Lhca and Lhcb proteins diverged before the separation of different Lhca and Lhcb complexes. Thus, Lhca and Lhcb proteins are separate groups within the Lhc family, suggesting they have specific properties and

```
Lhca1   -------MASNSLMSCGIAAVYPS------LLSSS-------K-SKFVSA
Lhca2   --------MASSLCASSAIAAISS---PSFLGGKK---LRLKKKLTVPAV
Lhca3   --MAAQALVSSSLTSSVQTARQIFGSKP--VASAS------QKKSSFVVK
Lhca4   --------MATVTTHASASIFRPCTSKPRFLTGSS---GRLNRDLSFTSI
Lhca5   --------MAVVLRGGITGGFLHHR-R----DASS----VITRRISSVKA
Lhca6   MAFAIASALTSTLTLSTSRVQNPTQRRPHVVASTSSTGGRLMRERLVVVR
                 :                      . .       :

Lhca1   GVPLPNAGNVGRIRMAAHWMPGEPRPAYLDGSAPGDFGFDPLGLGEVPA-
Lhca2   SRPDASVRAVAADPDRPIWFPGSTPPEWLDGSLPGDFGFDPLGLSSDPD-
Lhca3   A---AAAPPVKQGANRPLWVASSQSLSYLDGSLPGDYGFDPLGLS-DPEG
Lhca4   G-SSAKTSSFKVEAKKGEWLPGLASPDYLTGSLAGDNGFDPLGLAEDPE-
Lhca5   A---GGGINPTVAVERATWLPGLNPPPYLDGNLAGDYGFDPLGLGEDPE-
Lhca6   AGKEVSSVCEPLPPDRPYGSLVALHLNWLDGSLPGDFGFDPFGLGSDPD-
        .                          :* *. .** ****:**. *

Lhca1   -----NLERYKESELIHCRWAMLAVPGILVPEALGYGNWVKAQ---EWAA
Lhca2   -----SLKWNVQAEIVHCRWAMLGAAGIFIPEFLTKIGILNTP---SWY-
Lhca3   TGGFIEPRWLAYGEIINGRFAMLGAAGAIAPEILGKAGLIPAETALPWFQ
Lhca4   -----NLKWFVQAELVNGRWAMLGVAGMLLPEVFTKIGIINVP---EWY-
Lhca5   -----SLKWYVQAELVHSRFAMLGVAGILFTDLLRTTGIRNLP---VWY-
Lhca6   -----TLKWFAQAELIHSRWAMLAVTGIIIPECLERLGFIENF---SWY-
               .   ..*::: *:***...* : .: :   .         *

Lhca1   ----LPGGQATYLGNPVPWGTLPTILAIEFLAIAFVEHQRSMEK-DP---
Lhca2   -----TAGEQEY------FTDKTTLFVVELILIGWAEGRRWADIIKPGSV
Lhca3   TGVIPPAGTYTY------WADNYTLFVLEMALMGFAEHRRLQDWYNPGSM
Lhca4   -----DAGKEQY------FASSSTLFVIEFILFHYVEIRRWQDIKNPGSV
Lhca5   -----EAGAVKFD-----FACTKTLIVVQFLLMGFAETKRYMDFVSPGSQ
Lhca6   -----DAGSREY------FADSTTLFVAQMVLMGWAEGRRWADLIKPGSV
              .*   :      :    *::. ::  : :.* :*  :  .*

Lhca1   EKK------------K-----YPGG-AFDPLGYSK-DPKKLEELKVKEIK
Lhca2   NTD-PVFP--NNKLTG-TDVGYPGGLWFDPLGWGSGSPAKLKELRTKEIK
Lhca3   GKQ--YFLGLEKGLAGSGNPAYPGGPFFNPLGFGK-DEKSLKELKLKEVK
Lhca4   NQD-PIFK--QYSLPK-GEVGYPGG-IFNPLNF-----APTQEAKEKELA
Lhca5   AKEGSFFFGLEAALEG-LEPGYPGGPLLNPLGLAK-DVQNAHDWKLKEIK
Lhca6   DIE-PKYP--HKVNPK-PDAGYPGGLWFDFMMWGRGSPEPVMVLRTKEIK
          .                  ****  :: :             : **:

Lhca1   NGRLALLAFVGFCVQQSAYPGTGPLENLATHLADPWHNNIGDIVIPFN-
Lhca2   NGRLAMLAVMGAWFQH-IYTGTGPIDNLFAHLADPGHATIFAAFTPK--
Lhca3   NGRLAMLAILGYFIQG-LVTGVGPYQNLLDHLADPVNNNVLTSLKFH--
Lhca4   NGRLAMLAFLGFVVQH-NVTGKGPFENLLQHLSDPWHNTIVQTFN----
Lhca5   NGRLAMMAMLGFFVQA-SVTHTGPIDNLVEHLSNPWHKTIIQTLFTSTS
Lhca6   NGRLAMLAFLGFCFQA-TYTSQDPIENLMAHLADPGHCNVFSAFTSH--
        *****::*.:*  .*    .  .* :**  **::* : .:   .
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Fig. 4. (A) Alignment of protein sequences of Lhca1–6 from *Arabidopsis thaliana*. Chl binding residues are indicated in bold.

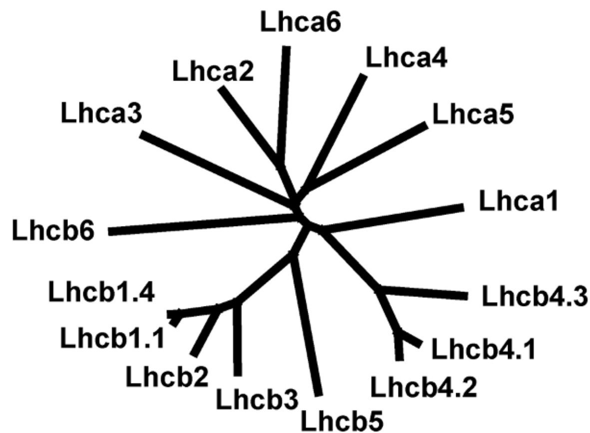

Fig. 4. (B) An unrooted cladogram of deduced protein sequences of Lhc proteins of *A. thaliana*.

functions, probably adapted to the characteristics of the photosystems that they serve.

B. Differential Characterization of Individual Lhca Gene Products

The first evidence for the presence of a Chl *a*/*b* antenna in higher plants PS I came from the work of Mullet et al. (1980b), who purified PS I complexes that differed in antenna size and in the presence of four polypeptides with molecular mass between 20 and 24 kDa, thereafter identified as the outer antenna system of PS I.

To purify the individual Lhca complexes and to determine their biochemical and spectroscopic characteristics, different approaches have been used: purification of LHCI from plants, reverse genetics, and refolding *in vitro*. In the following section, the main steps in the determination of the properties of the individual Lhca complexes are summarized.

1. Fractionation of LHCI – Native LHCI

a. Purification

LHCI complexes were first purified from pea by Haworth et al. (1983) using sucrose gradient ultracentrifugation. The authors isolated a fraction containing four polypeptides with molecular weights between 20 and 24 kDa, and with a Chl *a*/*b* ratio of 3.7. In the following years, several attempts to further purify LHCI complexes were performed using different methods including sucrose gradient ultracentrifugation (Kuang et al., 1984; Lam et al., 1984b; Bassi and Simpson, 1987; Ikeuchi et al., 1991a; Schmid et al., 2002b), nondenaturing electrophoresis (Kuang et al., 1984; Lam et al., 1984a; Bassi et al., 1985; Dunahay and Staeheli, 1985; Vainstein et al., 1989; Knoetzel et al., 1992; Preiss et al., 1993), and perfusion chromatography (Tjus et al., 1995).

In 1984 Lam et al. (1984a) purified two Lhca fractions from spinach by sucrose gradient ultracentrifugation. The upper band contained the 22 and 23 kDa polypeptides (Lhca2 and Lhca3), while the lower was enriched in the 20 kDa polypeptides (Lhca1 and Lhca4). The fractions showed similar Chl *a*/*b* ratio (3.5 ± 0.5), but differed substantially in their spectroscopic properties, with the upper band emitting at 680 nm and the lower and emitting at 730 nm. On the basis of these results, LHCI fractions were named according to their emission peaks at low temperature: LHCI-730 and LHCI-680 (Bassi et al., 1985) and this nomenclature was used in most of the papers that followed this work. From these early experiments it was concluded that the red forms were associated with the 20 and 21 kDa polypeptides (Lhca1 and Lhca4), rather than with the heavier subunits (Lhca2 and Lhca3). The polypeptide composition of LHCI-680 and LHCI-730 varied, however, in the different preparations. LHCI-730 in some preparations contained only Lhca1 and Lhca4 (Knoetzel et al., 1992; Tjus et al., 1995; Schmid et al., 2002b), while in others all four Lhca polypeptides were present (Bassi et al., 1985; Bassi and Simpson, 1987; Ikeuchi et al., 1991b). Typically, the LHCI-730 fraction had an absorption peak in the Q_y region of Chl *a* at 670–676 nm and a 77 K fluorescence emission peak at 730 nm. LHCI-680 was enriched in Lhca2 and Lhca3 polypeptides; it showed a lower sedimentation rate in a sucrose gradient and a higher mobility by nondenaturing electrophoresis (green gels) as compared to LHCI-730, indicating a lower aggregation state. LHCI-680 had an absorption peak in the Q_y region of Chl *a* at 668–670 nm and a 77 K fluorescence emission peak at 678–680 nm (Lam et al., 1984a; Knoetzel et al., 1992; Tjus et al., 1995). Exceptions to this pattern suggested a further grade of heterogeneity among the individual gene products: the "LHCI-680" isolated by Bassi and Simpson (1987) from barley contained a single polypeptide, Lhca2, and had a Chl *a*/*b* ratio of 2.0. Its absorption peak was at 674 nm and its fluorescence emission peaked at 690 nm. The LHCI-680 preparation described by Ikeuchi et al. (1991b), was enriched in Lhca2 and Lhca3. Whereas showing the maximum fluorescence emission occurred at 680 nm, it had a prominent shoulder above 700 nm suggesting the presence of red-shifted spectral forms in either Lhca2 or Lhca3. In other reports, the fractionation of the PS I–LHCI complex did not yield a LHCI-680

Table 1. Pigment composition of LHCI

Plant material	Fraction	Chl *a*/*b*	Chl tot	Viola	Lutein	β-car	References
Pea	LHCI	3.7					Haworth et al. (1983)
	680/730	3.5					Lam et al. (1984a)
Spinach	680	3.1					Palsson et al. (1995)
	730	3.2					Palsson et al. (1995)
Barley	680[a]	2.0					Bassi and Simpson (1987)
	730	2.2					Bassi and Simpson (1987)
Tomato	680	2.5	8.99	0.43	0.92	0.4	Schmid et al. (2002b)
	730	2.57	7.17	0.39	0.84	0.18	Schmid et al. (1997)
		2.48	11.41	0.54	0.99	0.42	Schmid et al. (2002b)
Maize	LHCI	3.8	10	0.55	1.2	0.4	Croce and Bassi (1998)
	680	4–5					Vainstein et al. (1989)
	730	2–2.5					Vainstein et al. (1989)
	Lhca2/Lhca3	3.8	10	0.5–0.6	1.1–1.2	0.5–0.65	Croce (1997)
	Lhca1/Lhca4	3.6–3.8	10	0.6–0.7	1.3–1.4	0.36–0.4	Croce (1997)
Arabidopsis	LHCI	3.3	10	0.6	1.1	0.45	Croce et al. (2002)
Spinach[b]		3.0	92	7	12	8	Damm et al. (1990)

Data shown were obtained with different purification methods and from different plant species (see section II.B).
[a] This preparation contained only Lhca2.
[b] Data were obtained by difference between the pigment composition of PS I-200 and PS I-core.

fraction. Haworth's preparation (1983) showed an emission at 730 nm and contained all four Lhca polypeptides. Also, the LHCI preparation by Croce et al. (1998) did not contain a LHCI-680 fraction but only a LHCI-730 fraction in a dimeric aggregation state. From the latter preparation, two emissions were resolved, peaking at 702 and 730 nm (Ihalainen et al., 2000). It was suggested that the LHCI-680 fraction contains complexes that are not in their fully native state. This is consistent with the blue-shifted absorption peaks (668–670 nm) and the fluorescence emission from partially disconnected Chls (Palsson et al., 1995) that have longer fluorescence lifetimes with respect to LHCI-730. Thus, it appears that Lhca2 and Lhca3 can migrate either as monomers, in the LHCI-680 fraction, or as dimers, in the LHCI-730 fraction, exhibiting spectroscopic differences depending on aggregation state: a longer lifetime emission and a blue-shifted absorption in LHCI-680 with respect to LHCI-730. This might be due to higher sensitivity to detergent treatment of Lhca2 and Lhca3 with respect to Lhca1 and Lhca4, as suggested by Lam et al. (1984a).

An attempt to purify Lhca2 and Lhca3 in the native state was made by Croce et al. (Croce and Bassi, 1998; Croce et al., 2002) using nondenaturing IEF. In this work, fractions containing dimeric complexes were purified either enriched in Lhca2/Lhca3 or in Lhca1/Lhca4. Red emission forms were retained, not only in Lhca4 containing fractions, but also in those in which Lhca2/Lhca3 was predominant, thus suggesting that one or both of these two subunits also accommodate red-shifted Chl forms.

b. Pigment Content

The purification of individual Lhca complexes in their native state by classical biochemical methods has proven very difficult due to the strong association between the external antenna and the core complex. Thus, harsh detergent treatment is required for dissociation. Nevertheless, information about the average biochemical and spectroscopic characteristics of the LHCI complexes was obtained in several plant species.

A summary of LHCI preparations is presented in Table 1. The pigment composition of the complexes is also reported.

The Chl *a*/*b* ratio values obtained from the different preparations vary considerably, with tomato and barley showing the lowest values, 2.5, and maize and pea the highest, 4. Both the methods of preparation and the intrinsic differences in the Chl *b* content of LHCI from different species might be the cause for these differences as judged by the finding that the same procedures applied to maize and Arabidopsis yielded *a*/*b* ratios of 3.8 and 3.3, respectively (Croce et al., 2002). Preparations involving the use of Triton X-100 appear to undergo partial Chl loss. More consistent are the data on the carotenoid composition: Lhca complexes bind violaxanthin and lutein, similar to Lhcb proteins (Bassi et al., 1993). However, they do not bind neoxanthin. β-carotene, which is not bound to Lhcb proteins, instead appears as a minor but genuine chromophore for Lhca proteins.

Thus, biochemical fractionation, although not yielding purified, individual, undenatured, Lhca gene

products, allowed characterization of the overall properties of LHCI. Moreover, evidence was provided for heterogeneity of the four Lhca components with respect to both pigment composition and enrichment in red-shifted absorption forms.

2. Reverse Genetic and Chlorina Mutants

An alternative approach to clarify the properties of Lhca complexes has been the analysis of plants lacking one or more Lhca gene products. The barley *Chlorina* mutant collection (Simpson et al., 1985) has first been used, followed by knockout mutants or antisense plants.

The *Chlorina f2* mutant lacking Chl *b* was analyzed by Mullet et al. (1980a) to support Chl *b* binding to LHCI complexes and their content in red emission forms. The preferential failure of *Clo-f2* to accumulate Lhca4 led to the conclusion that the red forms are associated to this subunit (Bossmann et al., 1997; Knoetzel et al., 1998). This suggestion was supported by Zhang et al. (1997), who produced antisense Arabidopsis plants lacking Lhca4, showing a 6 nm blue shift in the fluorescence emission peak. Antisense plants lacking Lhca2 and Lhca3 (Ganeteg et al., 2001) led the authors conclude that absorption forms at 695 and 715 nm, connected to emissions at 702 and 735 respectively, were associated to Lhca2 and Lhca3. Thus the use of mutant plants lacking one or more Lhca proteins showed to be valuable in establishing the relation between gene products and the associated absorption and fluorescence forms, thereby overcoming the problems due to the partial denaturation of pigment–proteins during purification.

3. Recombinant Proteins

A third approach to the study of PS I antenna system has been the *in vitro* production of Lhca holoproteins by overexpression of the apoproteins in bacteria and reconstitution *in vitro* with purified pigments. This approach, first introduced by Plumley and Schmidt (1987), exploits the ability of Lhc proteins to fold *in vitro* in the presence of Chls and carotenoids. This procedure was first applied to Lhcb proteins, which can be purified more easily than Lhca subunits from thylakoids. Comparison of native and recombinant proteins showed that reconstitution *in vitro* yielded complexes indistinguishable by biochemical and spectroscopic analysis (Giuffra et al., 1996; Pascal et al., 2001; Croce et al., 2003).

In 1997 Schmid et al. (1997) reconstituted Lhca1 and Lhca4 using tomato genes and showed that the two complexes, although homologous, had distinct properties, with Lhca4 emitting at 730 nm and Lhca1 at 686 nm. These experiments provided a direct demonstration that the red-most forms were associated to Lhca4. The same procedure was later applied to Lhca2 and Lhca3 from tomato (Schmid et al., 2002b) and to the four Lhca gene products from Arabidopsis (Croce et al., 2002; Castelletti et al., 2003). It was shown that red-shifted forms are a common characteristic of Lhca proteins, their amplitude and wavelength of emission increasing in the order Lhca1 > 2 > 3 and 4 (Castelletti et al., 2003).

Table 2. Pigment composition of reconstituted Lhca complexes from *Arabidopsis thaliana*

Sample	Chl *a*	Chl *b*	Viola	Lutein	β-car
Lhca1	8 ± 0.04	2 ± 0.04	1.05	1.81	–
Lhca2	6.5 ± 0.2	3.5 ± 0.2	0.47	1.52	–
Lhca3	8.6 ± 0.2	1.4 ± 0.2	0.66	1.62	0.57
Lhca4	7.0 ± 0.2	3 ± 0.2	0.5	1.5	–

Data shown are from Castelletti et al. (2003).

The pigment content of the four Lhca complexes from Arabidopsis is reported in Table 2. All Lhca were estimated to coordinate 10 Chls per polypeptide, while differing sensibly in their Chl *a* to Chl *b* ratio. In particular, a higher Chl *b* content was found in Lhca2 (Chl $a/b = 1.8$), while the lowest was found in Lhca3 (Chl $a/b = 6$). The carotenoid content was found to be either two (in Lhca2 and Lhca4) or three (in Lhca1 and Lhca3). Reconstitution *in vitro* confirmed that neoxanthin is not a component of the antenna system of PS I. In fact, although present in the reconstitution mixture during refolding, it was never bound in the complexes. Lhca3 has the peculiar feature of binding β-carotene in its monomeric state (Croce et al., 2002; Castelletti et al., 2003). While the results on tomato Lhca differ slightly, the trend in both Chl *a*/*b* and carotenoids binding (Schmid et al., 2002b) is consistent with the data found using Arabidopsis genes. Reconstitution in the absence of individual pigment species revealed that Lhca1 and Lhca3 need Chl *a*-only for folding, while Chl *b* is, in addition, essential for the stability of Lhca2 and Lhca4 (Schmid et al., 2002b), consistent with the different affinity of the complexes for the two Chl species.

The possibility to prepare homogeneous preparations of each Lhca complex allowed their spectroscopic properties to be determined. The analysis of the emission spectra at low temperature showed that all Lhca complexes contained emission forms at wavelengths longer than 700 nm. In particular Lhca4 showed an emission maximum at 733 nm, Lhca3 at 725 nm, Lhca2

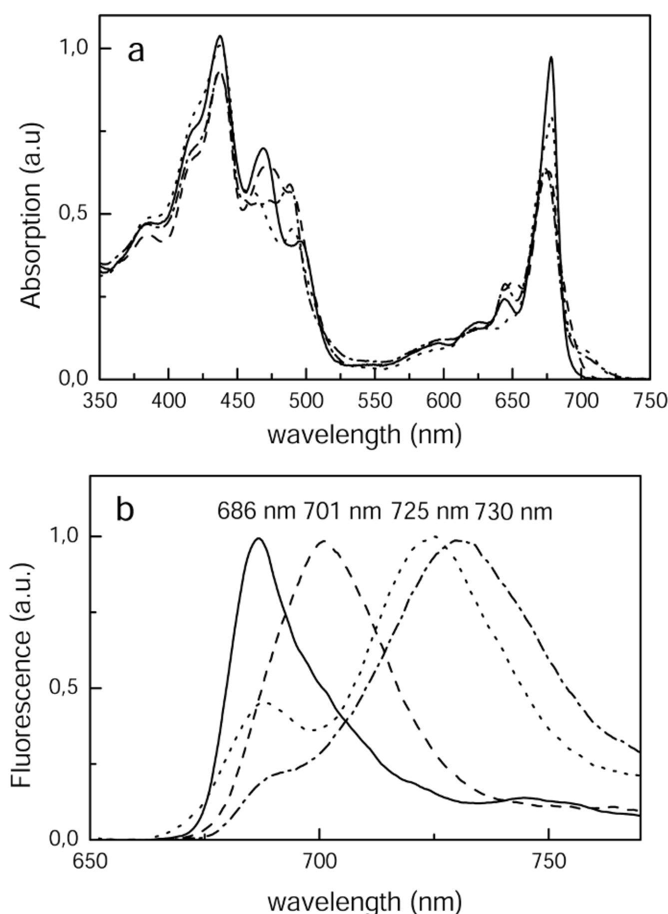

Fig. 5. (a) Absorption and (b) fluorescence emission spectra at 77 K of recombinant Lhca complexes: Lhca1 (solid), Lhca2 (dashes), Lhca3 (dots), Lhca4 (dash-dots). All genes are from *Arabidopsis thaliana*. Emission maxima are also reported.

at 702 nm. The Lhca1 emission peak was at 690 nm but a shoulder was also present at 701 nm (Fig. 5b). The absorption spectra of individual Lhca proteins (Fig. 5a) demonstrated that each of them has peculiar characteristics and a different distribution of the absorption forms. The Q_y absorption peaks of Lhca1, Lhca2, and Lhca3 are found at around 680 nm, strongly red shifted with respect to Lhcb complexes. This is not the case for Lhca4, for which the Q_y absorption peak was found at 676 nm (Fig. 5b).

A comparison between the pigment organization in Lhca and Lhcb complexes can be had by analyzing the CD and LD spectra of the complexes. A red shift in both CD and LD is observed for Lhca complexes as compared to Lhcb, which indicates that the red forms contribute to the dichroic spectra. Interestingly, in the region in which carotenoids absorb, the spectra showed larger differences, thus supporting the hypothesis that the xanthophylls in Lhca complexes are differently organized/oriented than in Lhcb complexes (Croce et al., 2002; Castelletti et al., 2003; Morosinotto et al., 2003).

In vitro reconstitution resulted in a major contribution to the detailed knowledge of the biochemical and spectroscopic characteristics of individual Lhca complexes. It was clear that the four Lhca complexes, despite their high sequence homology, strongly differ in their pigment binding affinity and in the distribution of the absorption forms. Strongly shifted fluorescence emissions were associated with both Lhca4 and Lhca3 monomers, while Lhca1 and Lhca2 had a limited shift of their red-most spectral forms.

III. Models of LHCI Polypeptides

A. Polypeptide Structure

The structure of one member of Lhc family, the main antenna complex of PS II, LHCII, has been solved using

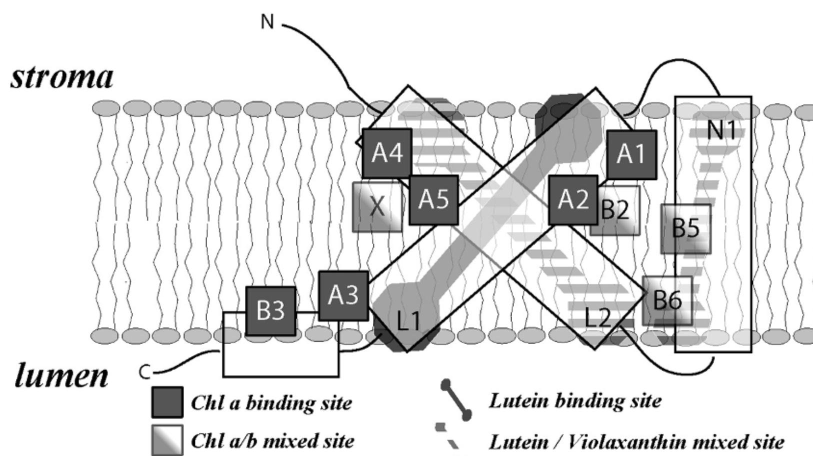

Fig. 6. Schematic 2D representation of Lhca structure, based on reconstitution experiments. Conserved Chl and xanthophyll binding sites are indicated, using the nomenclature proposed by Kühlbrandt et al. (1994). Residues directly coordinating chlorophylls are also indicated.

2D crystals and electron crystallography (Kühlbrandt et al., 1994). The high sequence homology between the members of the Lhc multifamily (Durnford et al., 1999) suggested that they share the same structural organization (Green and Durnford, 1996). This was confirmed by the structure of the PS I–LHCI complex, in which the 3D organization of Lhca polypeptides was solved. Superimposition of the structure of the transmembrane domain of Lhca complexes with LHCII showed an almost perfect match, with three transmembrane helices and one amphipatic helix exposed on the lumenal side of the membrane (Ben Shem et al., 2003). The N-terminus is exposed to the stroma and the C-terminus is exposed to the thylakoid lumen (Fig. 6; Kühlbrandt et al., 1994; Ben Shem et al., 2003).

Less information is at present available about the organization of loops connecting α-helices, which are not resolved in the 2D crystals of LHCII. An analysis of the primary structure reveals differences between the Lhc proteins in the length of the loops connecting the transmembrane helices. In this respect, Lhca1 is similar to Lhcb4, having the stromal and the lumenal loops of the same length (23–25 amino acids). Lhca2, Lhca3, and Lhca4 have longer stromal loops of 41 (Lhca2), 43 (Lhca3), and 36 residues (Lhca4), and shorter lumenal loops of 17 (Lhca2), 26 (Lhca3), and 17 residues (Lhca4), making these complexes more similar to Lhcb6. Structural data suggest that these differences do not influence the packing of the transmembrane domain, but might be involved in interactions with core complex subunits (Ben Shem et al., 2003).

B. Chlorophyll Binding

1. How Many Chls are Bound to Each Lhca Complex?

A pigment determination on a purified LHCI fraction containing all four Lhca complexes showed that there are, on average, 10 Chls per polypeptide (Croce and Bassi, 1998). This value was consistently found in fractions enriched in Lhca2–3 or Lhca1–4. In the PS I–LHCI structure (Ben Shem et al., 2003), the authors observed 56 Chls associated with the external antenna, 14 Chls per each Lhca complex. However, Lhca proteins appear to be embedded in a sort of “pigment bed” composed of Chls either tightly bound to Lhca proteins or bound at the interface between two neighbor Lhca polypeptides or filling the gap between the LHCI crescent-like structure and the PS I-core complex. A portion of the Chls loosely associated with LHCI thus appear to be prone to dissociation during solubilization, thus leaving 10 Chls bound to each Lhca polypeptide as determined from both the Chl-to-polypeptide ratio and the carotenoid to Chl ratio. The latter parameter, in particular, appears to be reliable due to the inner location of three carotenoid binding sites in Lhc proteins (Croce et al., 2002; Castelletti et al., 2003).

2. Chlorophyll Organization

From the structure of LHCII complex, eight amino acid residues were indicated as Chl ligands, while the

Table 3. Biochemical and spectroscopic properties of the Chls in each binding site for Lhcb1 and Lhca1

Site	Lhcb1	Lhca1
A1	Chl *a* (679 nm)	Chl *a* (nd)
A2	Chl *a* (681 nm)	Chl *a* (682 nm)
A3	Chl *a*/Chl *b* (662/650 nm)	Chl *a* (663–681 nm)
A4	Chl *a* (674 nm)	Chl *a* (678–679 nm)
A5	Chl *a* (674 nm)	Chl *a* (675–679 nm)
B3	Chl *a*/Chl *b* (665/650 nm)	Chl *a* (663 nm), traces Chl *b*
B5	Chl *b* (652 nm)	Chl *a*/Chl *b* 644/(668–686/644 nm)
B6	Chl *b* (652 nm)	Chl *a*/Chl *b* 644/(668–686/644 nm)
B1	Chl *a* (679.4 nm)	–
B2	Chl *b* (644 nm)	Chl *a*/Chl *b* (650/682 nm)
A6/A7	0.5 Chl *a*/1.5 Chl *b* (679/652 nm)	–
X[a]	–	Chl *a*/Chl *b* (644/ND)

Data shown are from Remelli et al. (1999) and Morosinotto et al. (2002). The site nomenclature used here is derived from the LHCII nomenclature proposed by Kühlbrandt et al. (1994). It indicates the position of the chlorophyll in the structure. The A and B prefix is not meant here as indication of the Chl *a*/Chl *b* occupancy of the site.

[a] This site seems do not be present in Lhcb1 complex. In Lhca this Chl is lost together with Chl A5.

mode of binding of the remaining four Chl molecules remained unclear. Mutation analysis confirmed that all eight Chl binding residues were indeed able to coordinate the central Mg^{2+} of the Chls in LHCII. It was suggested that the additional four Chls were positioned by interaction with other chromophores, although the involvement of residues in the intra-helices loops could not be excluded (Kühlbrandt et al., 1994; Remelli et al., 1999). Primary structure comparison between LHCII and the Lhca complexes showed that all of the Chl binding residues present in LHCII are conserved in Lhca proteins, with a few substitutions: (i) Chl B6 is coordinated by a Gln in LHCII, while this position is occupied by a Glu in all Lhca proteins; (ii) an Asn residue is present at site A5 in Lhca3 and Lhca4, while in LHCII as well as in all other antenna complexes the ligand for Chl A5 is a His. Mutation analysis confirmed that these residues coordinate Chls as well as all other conserved amino acids (Morosinotto et al., 2002, 2003).

To assign the Chl occupancy to the individual sites and to determine the spectroscopic properties of each Chl, a detailed study of the pigment organization was performed by mutation analysis on Lhca1 (Morosinotto et al., 2002). The results are summarized in Table 3, in which the attribution of chromophores to different sites in Lhca1 is compared to that in Lhcb1.

The results indicate that the occupancy of most of the sites in Lhca1 is similar to Lhcb1, the major differences being due to the increased affinity for Chl *a* vs. Chl *b* of the sites showing mixed Chl *a* and Chl *b* occupancy. Interestingly, in Lhca1 most of the mutations caused the loss of more than one pigment in a manner different from that observed in Lhcb1 and Lhcb4, while several interactions between chromophores were spectroscopically detected in LHCI (Bassi et al., 1999; Remelli et al., 1999). This different pattern of responses suggests that pigment–pigment interactions are stronger in Lhca proteins as compared to Lhcb proteins. The crystal structure shows that the plane of the tetrapyrrole is differently oriented in at least three Chls within Lhca complexes as compared to LHCII, namely Chls in sites B5 (or A5), A7, and A3 (Ben Shem et al., 2003).

C. Carotenoid Binding

The carotenoid organization in Lhca1 was not resolved in the crystal structure, thus information is restricted to data from mutational analysis. Most of the mutations to the Chl binding residues also yielded loss of carotenoid molecules. This was useful for localizing the xanthophyll binding sites within Lhca1 and for determining their affinity for ligands. Three carotenoids are tightly bound to Lhca1 as well as to Lhcb1, and they are likely to be located at the same three binding sites, namely L1 and L2, nearby helices A and B, and N1 near helix C. The affinity of each site for the individual xanthophyll species is not the same: neoxanthin, which is accommodated in the site N1 of Lhcb1 (Croce et al., 1999) is absent in Lhca1 and is substituted by violaxanthin and lutein. Lutein is conserved as a ligand for site L1 in all Lhc complexes analyzed thus far. However, while the occupancy of site L1 is essential for Lhcb protein folding, in Lhca1, lutein is lost on mutating site A3 without destabilizing the complex (Bassi et al., 1999; Remelli

et al., 1999). Site L2 accommodates both lutein and violaxanthin with similar affinities.

On the basis of the results of the mutational analysis, a model for the chromophore organization in Lhca1 can be drawn (Fig. 6).

Preliminary results on the mutational analysis of Lhca2, Lhca3, and Lhca4 suggest that all Lhca proteins share a similar pigment organization with the exception of the number of bound xanthophylls: two in Lhca2 and Lhca4, three in Lhca 1 and 3. Lutein is in site L1, while site L2 is occupied by both lutein and violaxanthin. Lhca3 was shown to coordinate also β-carotene (Schmid et al., 2002b; Castelletti et al., 2003), but its binding site is not yet clear.

The structure of Lhca complexes is similar to LHCII with respect to both apoprotein and chromophores organization. However, differences can be observed in the affinity of the pigment binding sites for the individual chromophores. Small differences in the pigment organization can be detected both by mutational analysis and by X-ray diffraction, suggesting that the peculiar spectroscopic characteristics of Lhca complexes might be due to small changes in inter-chromophore distance and orientation, particularly in the domain including the stromal side of helices A and C.

IV. Dimerization of Lhca Proteins

It is now accepted that all Lhca complexes are present in dimeric form *in vivo* (Croce and Bassi, 1998; Croce et al., 2002). This suggestion was first made by Kuang et al. (1984) and by Bassi (1985) from their apparent mobility as a 40 kDa band in nondenaturing electrophoresis. A similar suggestion was also made based on sucrose gradient ultracentrifugation (Ikeuchi et al., 1991a) and by nearest-neighbor analysis (Jansson et al., 1996) that yielded cross-linking products between 40 and 45 kDa.

The suggestion that Lhca1 and Lhca4 form heterodimers (Knoetzel et al., 1992) was later confirmed by *in vitro* reconstitution (Schmid et al., 1997). In the Lhca1–4 heterodimer there is complete energy transfer between Lhca1 and Lhca4, as demonstrated by the single 733 nm emission peak in the spectrum of the dimer. The CD and the LD spectra of the dimer also showed that the dimerization influenced the pigment organization, especially of Chl *b* (Schmid et al., 1997; Croce et al., 2002). Moreover, the heterodimer was more stable to heat denaturation than the two monomers. Dimerization also involved the binding of β-carotene, probably at the subunit interface (Croce et al., 2002).

Consistent with the *in vitro* data, analysis of *Chlorina* mutants (Knoetzel et al., 1998) and of Lhca4 antisense plants (Zhang et al., 1997) showed that although Lhca1 is maintained in the absence of Lhca4, its stoichiometry with respect to PS I RC is decreased, suggesting that they form dimers *in vivo*.

The results on Lhca2 and Lhca3 are less clear: they do not dimerize *in vitro* (Schmid et al., 2002b) but antisense plants lacking either Lhca2 or Lhca3 showed relevant reduction of Lhca3 and Lhca2, respectively, thus suggesting that the two subunits are connected (Ganeteg et al., 2001). Moreover, partial purification of LHCI by preparative isoelectrofocusing yields both dimers enriched in Lhca1–4 and in Lhca2–3, suggesting their association (Croce et al., 2002). Evidence for Lhca2 homodimers was obtained by analyzing a native LHCI preparation containing all Lhca complexes: two different emissions, at 702 and 733 nm were detected (Ihalainen et al., 2000), which is predicted only if dimers of Lhca2 cannot transfer energy to Lhca3, whose red-most transition is at far lower energy (Castelletti et al., 2003).

The dimerization of Lhca1 and Lhca4 has been studied *in vitro*, producing proteins modified by deletions or point mutagenesis. It has been shown that the Trp residue at position 4 of Lhca1 is involved in dimerization together with an additional Trp residue localized at the C-terminus of the same protein. Lhca4 C- and N-termini are not involved in strong interactions with Lhca1 since Lhca1–4 dimerization was broken only after complete deletion of the N- and C-terminal sequences (Schmid et al., 2002a). This is consistent with the X-ray data showing that Lhca1 binds to the C-helix and to the lumenal loop of Lhca4.

We can conclude that Lhca complexes are present as heterodimers *in vivo* with the Lhca1–4 pair being more stable than the Lhca2–3 dimer. More experiments are needed to elucidate the interaction between Lhca2 and Lhca3 and to determine the spectroscopic characteristics of this heterodimer.

V. PS I–LHCI Stoichiometry

The number of antenna proteins bound to PS I has, for long time, been a matter of debate. It was believed that there were eight Lhca per P700. This figure was derived from EM analysis (Boekema et al., 1990), and it was in good agreement with pigment stoichiometry data that attributed 80 Chls to the LHCI moiety and 10 Chls to each polypeptide (see above). However, a more detailed electron microscopic analysis suggested

that the surface occupied by LHCI in projection with the PS I–LHCI complex could accommodate four to five Lhca per reaction center (Boekema et al., 2001b). The 3D structure of the PS I–LHCI complex supports a stoichiometry of four Lhca per PS I. The structure also allowed the discrepancy between the pigment stoichiometry and the structural data to be explained. In fact, linker Chls are present in between the Lhca complexes and the core and in between the individual Lhca subunits. These Chls could be lost upon separation of LHCI from the core, thus influencing estimations of stoichiometry based on the pigment content of individual polypeptides.

Thus, four Lhca complexes are present in the PS I–LHCI supercomplex, and are connected to the core by linker Chls that are lost upon dissociation of the LHCI fraction from the core moiety.

VI. Energy Transfer

Analysis of the excitation spectra of Lhca complexes reveals that the carotenoid transfers energy to the Chls with 65%, efficiency, a value lower than in Lhcb complexes (70–80%, Croce et al., 2002; Castelletti et al., 2003). The energy transfer pathways in Lhca complexes are reviewed in Karapetyan et al. (this volume, Chapter 13), where the reader is provided more details. Here, we stress that the value obtained from time-resolved data indicate that the energy transfer within LHCI complexes is very similar to that observed within Lhcb complexes. The only difference is represented by the energy transfer component in the red absorbing forms, which takes place in approximately 5 psec. We note that a similar lifetime has been associated with the Chl *a*–Chl *a* equilibration in LHCII and in CP29 (Van Amerongen and van Grondelle, 2001; Croce et al., 2003). The energy transfer between monomers within the dimeric complex is slower than the energy transfer between individual subunits in LHCII trimers (30 psec vs. 12 psec, Kleima et al., 1997). These results suggests that the pigment organization in Lhca complexes is not very different compared to Lhcb with the exception of the presence of the red forms, while the monomers in the dimer seem to be less strongly associated with respect to the trimer of LHCII.

VII. On the Origin of Red Absorption Forms

Following characterization of individual LHCI components, the elucidation of the structural features responsible for the huge shift in the Chl fluorescence emission has been the target of research on LHCI. In higher plant photosynthetic complexes, the Q_y absorption band of Chl is in the 640–660 nm range for Chl *b* and between 660 and 682 nm for Chl *a*. In the case of the red forms, the Chls involved are expected to absorb above 700 nm, and are thus energetically far apart from the bulk Chls. It has been proposed that the red forms represent the low energy band of an excitonic interaction between two or more Chl molecules (Gobets et al., 1994). In the following we will address some detailed questions about red forms:

A. Which complex(es) coordinate the red Chls?
B. Which are the absorption characteristics of the red forms?
C. How many Chls absorb in the red?
D. Which are the Chls involved?
E. Where are the red Chls located within the Lhca complexes?
F. What is the role played by the protein in modulating the absorption of the red Chls in the four Lhca complexes?
G. What mechanism is responsible for the large absorption shift of the red forms as compared to the bulk Chls?

A. Which Complexes Coordinate the Red Chls

The dissociation of PS I-200 into PS I-core and LHCI complexes resulted in the conclusion that the most red forms are associated with the outer antenna system in higher plants. This is in contrast with other organisms, in which the red emission originates from Chls associated with the PS I-core [see Gobets and van Grondelle (2001) for a review].

In vitro reconstitution of individual Lhca complexes yielded the emission spectra of individual Lhca complexes, showing that the emissions at 4 K for Lhca4, Lhca3, Lhca2, and Lhca1 were at 733, 725, 702, and 690 nm, respectively (Castelletti et al., 2003). The Lhca1–4 heterodimer showed the same fluorescence emission as the Lhca4 monomer, indicating complete energy transfer from Lhca1 to the red forms of Lhca4 (Schmid et al., 1997).

B. Which are the Absorption Characteristics of the Red Forms?

While the red forms can be easy detected by fluorescence, especially at low temperature, a determination of

the absorption forms responsible for the red emission is not straightforward since the red pigments represent, at most, the 5% of the total absorption in the Q_y region (Ihalainen et al., 2000). Although these forms are expected to be energetically distant from the bulk Chls, it was not possible to detect a clear band, even at 4 K, due to the lack of structure in the spectra. This suggests that these forms are largely inhomogeneously broadened. From an early analysis of the 735 nm emission of chloroplasts (Butler et al., 1979) it was proposed that the origin of the red emission was an absorption band at 705 nm (Boardman et al., 1978). Site-selected fluorescence measurements on a PS I-200 preparations indicated 716 nm as the upper limit for the maximum of the band responsible for the long wavelength emission (Gobets et al., 1994). Similar work on a purified dimeric LHCI fraction shifted this estimation to 711 nm (Ihalainen et al., 2000). The bandwidth of the 711 nm absorption was suggested to be 356 cm^{-1} by Gaussian deconvolution of the red tail of LHCI absorption spectra, while site-selected fluorescence analysis showed that the contribution of the homogeneous and inhomogeneous broadening to the bandwidth were 210 and 290 cm^{-1}, respectively. Consistently, an electron–phonon coupling value of 2.7 was derived from fluorescence line-narrowing experiments (Ihalainen et al., 2003): the stronger coupling found in photosynthetic antenna complexes. From these values the FWHM of the red band in LHCI of 30 nm and the Stokes shift of 35 nm could be calculated.

Analysis of the reconstituted Lhca complexes at 77 K, by Gaussian deconvolution of difference absorption spectra (see below), suggested that the absorption is located between 700 and 705 nm for both Lhca3 and Lhca4 (Morosinotto et al., 2003). The origin band for the 702 nm emission of Lhca1 and Lhca2 complexes was located at 686–688 nm by comparison of the spectra of WT and point mutant proteins (Morosinotto et al., 2002, unpublished).

C. How Many Chls Absorb in the Red?

In LHCI preparations it was estimated that the red absorption represents 5% of the total absorption in the Q_y band, which translates to 0.5 Chls per complex or 1 Chl molecule per dimer. Analysis of the absorption spectra of reconstituted complexes at low temperature suggested that in both Lhca3 and Lhca4 the red tail represents roughly the absorption of one Chl molecule (Morosinotto et al., 2003), in agreement with the analysis on the native complex. Due to the difficulties associated with the determination of the actual band shape of the low energy absorption forms, this value has to be taken as a rough estimate. However, the value suggests that the Chls involved in the excitonic interaction that yield the red forms are not in the head-to-tail arrangement, but instead their dipole transition vectors are organized almost perpendicular to each other.

D. Which Chl Specie(s) is Involved in Red Forms?

Mukerji and Sauer (1990) observed that excitation of Chl *b* increased the amplitude of red emission as compared to excitation of Chl *a*, and thus concluded that Chl *b* is closely connected to red-shifted chromophores. A suggestion of a direct involvement of Chl *b* in the red forms was also made by Schmid et al. (2001) based on the observation that reconstitution of Lhca4 in the absence of Chl *b* caused a loss of red forms. However, Chl *a*-only Lhca3 (Castelletti et al., 2003) retained the red-shifted emission while Chl *a*-only Lhca4, although reducing the amplitude of red emission, still retained in part this spectral feature. It was shown that by increasing the Chl *b* content in Lhca4 above the control level, a progressive blue shift from 733 to 722 nm resulted in the emission peak. This result was interpreted as evidence that a Chl *a*–Chl *a* interaction was substituted by a Chl *b*–Chl *a* interaction, thus yielding a smaller emission shift due to the larger difference in the transition energy level of interacting monomers (see below). These results show that the interacting Chls responsible for red forms are in fact Chl *a* molecules, while suggesting that Chl *b* is possibly needed to maintain the conformation of chromophores responsible for the red forms.

E. Where are the "Red Chls" Located in the Lhca Architecture?

The mutation of Chl binding residues in Lhca complexes allows determination of the binding sites responsible for the red forms. Mutational analysis of Lhca1 (see section III.B) revealed that the 702 nm fluorescence emission component is lost on mutating sites A5, B5, and B6. On the basis of the occupancy of these sites (Chl *a* for A5, mixed site for B5 and Chl *b* for B6), it was proposed that the red emission originated from a Chl *a*–Chl *a* interaction between Chls located in sites A5 and B5, while the presence of a Chl in site B6 stabilized the conformation leading to the red forms. The mixed occupancy of site B5 explains the presence of the 690 nm fluorescence component: when Chl *b* is accommodated in site B5 the Chl *a*–Chl *a* interaction

that yields the red form is lost, yielding instead, the 690 nm bulk emission (Morosinotto et al., 2002).

The involvement of the A5 B5 domain in the origin of red forms was confirmed in all Lhca complex by constructing mutants at the Chl binding site A5, B5, and B6 (Morosinotto et al., unpublished). In all complexes the absence of Chl A5 and B5 led to the complete disappearance of red emission. B6 mutants, instead, have a slightly different phenotype, especially in Lhca3 and Lhca4: red emission is strongly reduced but not abolished.

From the analysis of the Lhca mutants two conclusions have been drawn: (i) the red forms originate from the same chromophores in all Lhca complexes; (ii) the model proposed for the origin of red forms by excitonic interactions of two Chl *a* molecules in the sites A5 and B5, proposed after the analysis of mutations in Lhca1 is probably maintained in all Lhca proteins.

An alternative hypothesis was recently proposed suggesting that a new Chl binding site, whose ligand residue would be a His belonging to helix C, is responsible for the red forms by establishing excitonic interactions with both Chl A5 and Chl B5 (Melkozernov and Blankenship, 2003). This hypothesis is not consistent with the previous report that Lhca3 has a strong red-shifted emission (Schmid et al., 2002b; Castelletti et al., 2003) and yet lacks the putative Chl binding His residue. Moreover, the recent structure of Lhca4 does not show the presence of an additional Chl coordinated to this particular residue (Ben Shem et al., 2003).

F. What is the Role of the Polypeptide Chain in Modulating the Absorption of the Red Chls in the Four Lhca Complexes?

Primary sequence analysis shows a strong homology between Lhca2 and Lhca4. Lhca1 and Lhca3 are also closely related. This clustering extends to the Chl *a*/*b* ratio and to the number of xanthophylls per polypeptide. However, the distribution of red forms is not consistent with the pattern being associated mainly with Lhca3 and Lhca4. Rather, it correlates with a specific substitution in the ligand of Chl A5: an asparagine in Lhca1 and Lhca3 versus a histidine in Lhca1–2 and in the rest of Lhcb proteins, which are depleted in red forms. A mutation at the A5 ligand (Asn > His) in Lhca3 and Lhca4 led to a loss of the red forms without affecting the pigment composition. The importance of the presence of Asn as a ligand in site A5 for the formation of red forms was confirmed by the reverse mutation on Lhca1: the His > Asn mutant gained red-shifted emission (Morosinotto et al., 2003).

G. What is the Mechanism Responsible for the Large Absorption Shift of the Red Forms Compared to the Bulk Chls?

The type of interaction between the chromophores responsible for the large absorption shift of the red forms has been investigated (Morosinotto et al., 2003) by comparing the absorption spectra of mutants lacking the red forms with the spectra of WT complexes. It was shown that in the His 97 mutant of Lhca4, two absorption forms at 682 and 703 nm were lost, while absorption at 675 nm was gained. This spectrum was interpreted as the loss of an excitonic interaction, having the high-energy term at 682 nm and the low energy contribution at 703 nm, while the 675 nm was representing the absorption of the noninteracting monomers. This was consistent with the analysis of the CD spectra (Morosinotto et al., 2003).

On this basis, it was proposed that part of the large shift of the red absorption band could be associated with the interaction energy (around 200 cm^{-1}). However, the energy associated with this interaction does not fully account for the large red shift of the absorption. Possibly, the presence of the Asn in this position directly influences the properties of the Chl ligand by changing the geometry of the tetrapyrrole or otherwise leading to a different folding of the protein domain that favors the red absorption.

The absence of the red emission in the Asn > His mutants of Lhca4 and Lhca3 was thus interpreted as the loss of the excitonic interaction between Chl A5 and Chl B5. Two parameters influence the interaction, the distance between the chromophores and the orientation of their dipole moments. LD spectra showed no changes in the chromophores orientation, thus suggesting that the substitution of Asn with His increased the distance between the two interacting Chls, thereby lowering the interaction energy (Morosinotto et al., 2003).

VIII. Lhca Proteins in *Chlamydomonas reinhardtii*

The first evidence for the presence of a Chl *b* containing antenna in LHCI was obtained in *Chlamydomonas reinhardtii* (Wollman and Bennoun, 1982). Evolutionary studies on Lhc complexes led to the conclusion that Lhca proteins diverged more than Lhcb during evolution (Durnford et al., 1999). The antenna complex of *C. reinhardtii* has recently been the subject of two electron microscopy studies (Germano et al., 2002;

Table 4. Lhca polypeptides identified in *Chlamydomonas* and their homologous in vascular plants

Chlamydomonas reinhardtii protein	Homologous in vascular plants	Nomenclature from Bassi et al. (1992)
Lhca1	Lhca1	p22.1
Lhca2	Lhca2	p19
Lhca3	Lhca3	p14.1
Lhca4	Lhca2 or Lhca4	p14
Lhca5	Lhca2 or Lhca4	p15.1
Lhca6	Lhca2 or Lhca4	p18.1
Lhca7	Lhca5	p15
Lhca8	Lhca5	p18
Lhca9	Lhca2	p22.2

The correspondence is derived from Stauber et al. (2003). The older nomenclature, used in previous work on *C. reinhardtii* (Bassi et al., 1992) is also reported.

Kargul et al., 2003). In these studies, the authors analyzed the structure of PS I–LHCI by single particle analysis and were led to the conclusion that in PS I between 11 and 14 Lhca subunits are bound to the core complex (Germano et al., 2002; Kargul et al., 2003). These data are consistent with a larger surface occupied by LHCI in *C. reinhardtii* as compared to spinach (Germano et al., 2002). Consequently, *C. reinhardtii* LHCI was shown to contain either seven (Bassi et al., 1992) or nine (Stauber et al., 2003) distinct polypeptides. These values, integrated with sequence data available in databases, allowed the identification of at least nine distinct polypeptides that compose the LHCI complex of PS I in *C. reinhardtii*. These polypeptides have been named Lhca1–9, but these names do not exactly reflect homology to higher plants genes. In particular, five different groups have been identified based on homology with vascular plants Lhca. This is summarized in Table 4 (Stauber et al., 2003).

The biochemical and spectroscopic properties of LHCI are known in less detail in *C. reinhardtii* than in vascular plants. Bassi et al. (1992) identified two LHCI populations, one emitting at 680 nm and the other at 705 nm. Similar purifications of LHCI from higher plants yielded LHCI-680 and LHCI-730 described above (see section II.B). Thus, the red forms of LHCI are not red shifted to the same extent as in vascular plants (Wollman and Bennoun, 1982).

Recently, Kargul et al. (2003) isolated PS I–LHCI complexes from *C. reinhardtii* showing a fluorescence emission maximum at 715 nm. Unfortunately it is not clear if this red emission originated from the antenna or from the core complex.

Pigment binding properties of LHCI of *C. reinhardtii* have not been analyzed in detail. The recent availability of protein sequences, however, shows that all eight Chl binding residues present in higher plant Lhcs are conserved in the *C. reinhardtii* proteins, strongly suggesting that these complexes bind at least eight Chls each. Xanthophyll binding has not been studied. Indirect evidence suggests that the xanthophyll binding properties of LHCI in *C. reinhardtii* are similar to those in vascular plants as deduced from the observation that *C. reinhardtii* mutants depleted in lutein, violaxanthin, and neoxanthin, thus leaving only zeaxanthin and β-carotene as carotenoid species available, were found to retain a stable LHCI. In these conditions Lhcb proteins were severely affected (Polle et al., 2001). The same phenotype was observed the Arabidopsis *npq2-lut2* mutant (Havaux et al., 2004).

From the comparison of Lhca sequences from Arabidopsis and *C. reinhardtii*, some interesting observations can be made. Lhca1 and Lhca3 from Arabidopsis and the corresponding genes from *C. reinhardtii* are highly homologous, while Lhca2 and Lhca4 from higher plants do not have closely related genes in *C. reinhardtii*. Moreover, it is interesting to note that in *C. reinhardtii* there exist two genes homologous to Lhca5 and one of them, Lhca8, is expressed at high levels (Stauber et al., 2003). This suggests that Lhca8 have a physiological function in *C. reinhardtii* that could possibly be maintained by the Lhca5 gene in Arabidopsis.

It has been observed that LHCI from *C. reinhardtii* showed reduced red forms as compared with higher plants. A possible explanation for these characteristics of algal LHCI can be found in the observation that genes highly homologous to higher plant Lhca4 and Lhca3, in fact, have His as the Chl A5 ligand. Asn however, is present at site A5 in three Lhca polypeptides of *C. reinhardtii* (Lhca2, Lhca4, Lhca9). Thus suggesting they may be responsible for the 705–715 nm fluorescence emission.

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