# The neoxanthin binding site of the major light harvesting complex (LHCII) from higher plants

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**Abstract The localisation of the xanthophyll neoxanthin within the structure of the major light harvesting complex (LHCII) of higher plants has been investigated by site-directed mutagenesis and spectroscopic methods. Mutation analysis performed on pigment binding sites in different helix domains leads to selective loss of neoxanthin for mutations on helix C thus localising this pigment between the helix C and helix A/B domains. Recombinant proteins binding two lutein molecules per polypeptide but lacking neoxanthin have been used in order to determine the contribution of neoxanthin to the absorption and linear dichroism spectra. The data were used to derive the orientation of the neoxanthin transition moment, lying in the polyene chain, which was thus determined to form an angle of 57 ± 1.5° with respect to the normal to the membrane plane where the protein is inserted. On the basis of these results we propose a model for the localisation of the carotenoid site in the LHCII structure which is still unresolved.**



*Key words:* Photosynthesis; Xanthophyll; Linear dichroism

## 1. Introduction

Light energy for photosynthesis of green plants is collected by an antenna system, composed of many homologous proteins belonging to the *Lhc* multigene family [1]. These pigment-proteins are organised around photosynthetic reaction centres to form supramolecular complexes embedded in the thylakoid membrane accounting for about 70% of the pigment involved in plant photosynthesis. LHCII is the most abundant light harvesting complex in higher plants and coordinates seven chlorophyll (Chl) *a* and five Chl *b* per polypeptide chain [2,3] whose function is light harvesting and excitation energy transfer to the photosystem (PS) II reaction centre. Carotenoids, also bound to LHCII, not only perform in light harvesting but also fulfil the essential role of protection against oxidation by either directly quenching singlet oxygen or preventing its formation from triplet chlorophyll. Significantly, Lhc proteins cannot fold in the absence of xanthophylls [4,5]. Three xanthophyll molecules are bound to LHCII in high affinity sites [6] while a fourth site, with lower affinity, can be occupied depending on the genotype and the physiological state of the plant [7,8]. Two of the sites to which xanthophyll molecules are bound have been located in the centre of the LHCII protein structure forming an internal cross-brace interacting with helices A and B [3]. These sites appear to be important in pigment-protein stabilisation [4,5] and were found to have the highest affinity for lutein [9]. Structural studies could not resolve the xanthophyll molecule bound to the third site, which is specific for neoxanthin. The binding of neoxanthin is a peculiar characteristic of LHCII. In fact, more than 90% of thylakoid neoxanthin is bound to LHCII trimers made by Lhcb1–3 gene products while Lhcb4 and Lhcb5 gene products only bind substoichiometric amounts of this pigment and Lhca1–4 as well as Lhcb6 proteins are unable to bind neoxanthin. Nevertheless, LHCII apoprotein can be refolded in vitro in the absence of neoxanthin thus yielding a pigment-protein binding two luteins per polypeptide chain, showing that occupancy of the neoxanthin site is not essential for protein folding [9]. In this study we have used in vitro reconstitution of LHCII protein overexpressed in bacteria either for mutation analysis of protein sequence or for selective modification of xanthophyll site occupancy. The former approach allowed localisation of neoxanthin close to the helix C domain, the latter was used for determination of the orientation of this pigment within the complex by using polarisation spectroscopy. On the basis of these data we propose a model for the organisation of the neoxanthin chromophore within LHCII pigment-protein structure.

## 2. Materials and methods

### 2.1. DNA constructions

The Lhcb1 expressing construction was previously described [9]. Plasmids were constructed using standard molecular cloning procedures [10]. Single point mutations were performed with Quickchange (Stratagene) or according to [11]. Bacterial hosts were *Escherichia coli* strains XL1Blue [12] and SG13009 [13]. The sequence was determined on both strands by the dideoxy method [14] by an automated apparatus (Applied Biosystems Model 377).

### 2.2. Isolation of overexpressed LHCII apoprotein from bacteria

LHCII was isolated from the SG13009 strain transformed with either of the two LHCII constructs following a protocol previously described [5,9].

### 2.3. Reconstitution of LHCII-pigment complexes

These procedures were performed as described by Giuffra et al. [15] with modifications reported in [9].

### 2.4. Purification of reconstituted LHCII

This was performed by ion exchange chromatography [15], followed by ultracentrifugation in a glycerol gradient (15–40% including 0.06% β-dodecyl maltoside (DM) and 10 mM HEPES pH 7.6; run was for

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*Abbreviations:* DM, β-dodecyl maltoside; Chl, chlorophyll; CP, chlorophyll-protein; c.m.c., critical micellar concentration; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-(hydroxy-ethyl)piperazine-*N*′-2-ethane-sulfonic acid; LHCII, light harvesting complex II; LD, linear dichroism; Lute (L), lutein; Neo (N), neoxanthin; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; SDS, sodium dodecyl sulphate; Viola (V), violaxanthin


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12 h at 60 000 rpm in a SW60 Beckman rotor) in order to eliminate the pigments unspecifically bound to the complex.

*2.5. Protein and pigment concentration*

The concentration of the LHCII apoprotein purified from *E. coli* inclusion bodies was determined by the bicinchoninic acid assay [16]. Chlorophyll concentration was determined by the method of Porra et al. [17]. HPLC analysis was as in [18].

*2.6. Spectroscopy*

Absorption spectra were obtained using a SLM-Aminco DW-2000 spectrophotometer at room temperature. Samples were in 10 mM HEPES pH 7.6, 0.06% DM, 20% glycerol. Fluorescence emission spectra were obtained at room temperature using a Jasco FP-777 spectrofluorimeter; bandwidth was 5 nm for excitation and 2 nm for emission. Chlorophyll concentration was about 10 μg/ml for absorption measurements and 0.01μg/ml for fluorescence measurements.

The 100 K absorption and linear dichroism spectra of the recombinant LHCII proteins were measured according to Haworth et al. [19] using samples oriented by the polyacrylamide squeezing technique as previously reported [20,21].

## 3. Results

Previous work on xanthophyll binding to LHCII showed that the neoxanthin binding site has different characteristics with respect to the lutein/violaxanthin sites, since it is highly selective for this xanthophyll species and is not needed for pigment-protein folding and stability. A search in the primary sequence of Lhcb1 deduced protein sequences for xanthophyll binding sites [22] revealed four motifs composed of hydrophobic stretches including one acidic residue on hydrophilic loops on both sides of helices A and B suggesting they are involved in binding lutein and violaxanthin in sites L1 and L2. No such conserved motifs were found elsewhere in the sequence suggesting neoxanthin might be bound to LHCII primarily by interactions with pigments rather than with the polypeptide backbone. This is supported by the recent finding of strong chlorophyll-neoxanthin interactions in LHCII [9]. In order to verify this hypothesis, we reasoned that if one or more chlorophyll molecules were important in its stabilisation, then selective removal of chlorophyll chromophores bound to individual sites, localised in different protein domains, could yield depletion of neighbouring neoxanthin. We thus proceeded to construct point mutations in maize *Lhcb1* cDNA [23] in positions previously identified as putative Chl binding sites [3]. Binding of Chl to these residues was confirmed by site-directed mutagenesis in the homologous protein CP29 [23]. The wild-type (WT) and mutant proteins were over-expressed in *E. coli* and reconstituted in vitro as previously described [9]. Pigment binding was characterised by a combined approach of HPLC analysis [25] and fitting of the acetone extracts with the spectra of purified pigments [6]. The WT LHCII protein reconstituted in vitro bound 12 chlorophylls per polypeptide with an *a*/*b* ratio of 1.4 and three xanthophyll molecules, lutein, violaxanthin and neoxanthin, in a relative ratio of 1.8:0.2:1.0. These values are essentially identical to those obtained with LHCII isolated from maize leaves [2,25]. The WT and mutant proteins were analysed by fluorescence emission spectroscopy in order to probe the correctness of the protein folding. Emission spectra were recorded between 600 and 800 nm with three different excitation wavelengths (440 nm, 475 nm and 500 nm) respectively exciting Chl *a*, Chl *b* and xanthophyll chromophores. In both WT and mutant proteins a single major peak was obtained at $683 \pm 2$ nm (not shown) thus demonstrating that both Chl *b* and xanthophylls are able to transfer excitation energy to Chl *a*. This strongly suggested that bound pigments had similar relative distances and orientations as found in the native protein.

Upon HPLC analysis LHCII proteins carrying mutations on helix A, helix B or helix D did not show significant changes in the ratio between neoxanthin and total carotenoids, which was conserved to a value of $0.35 \pm 0.03$, suggesting that xanthophyll complement was essentially the same. However, mutations in helix C Chl binding residues severely affected neoxanthin content. The Q to L mutation on residue 131, proposed to co-ordinate Chl $b_6$ in the LHCII structure [3], caused loss of half the bound neoxanthin and similar results were obtained with the E139L mutation affecting site $b_5$. The double mutant E139L/R142L showed a stronger effect since only traces of neoxanthin were found.

Biochemical and spectroscopic analysis of chlorophyll binding will be the subject of a companion paper. In this article we restrict the analysis to xanthophyll binding and conclude that since the removal of chlorophyll binding residues on helix C specifically induced neoxanthin loss, this xanthophyll is most probably bound to this domain. These results are summarised in Table 1.

Once the domain where neoxanthin is bound was located within the LHCII structure, further characterisation of this site required determination of carotenoid orientation with respect to the membrane plane. To this end we reconstituted

Table 1
Carotenoid composition of WT and mutant LHCII proteins

| Sample | Site-Helix | Pigment composition (mol/100 mol of Chl *a*+*b*) | | Neoxanthin/ total carotenoids |
|---|---|---|---|---|
| WT | / | Lutein | 42.36 | |
| | | Neoxanthin | 26.37 | 0.36 |
| | | Violaxanthin | 3.64 | |
| E65L/R185L | A4-A/B | Lutein | 39.6 | |
| | | Neoxanthin | 27.79 | 0.36 |
| | | Violaxanthin | 4.49 | |
| H68I | A5-B | Lutein | 51.36 | |
| | | Neoxanthin | 33.87 | 0.38 |
| | | Violaxanthin | 3.87 | |
| P82V | A6-B | Lutein | 44.79 | |
| | | Neoxanthin | 22.64 | 0.31 |
| | | Violaxanthin | 4.49 | |
| Q131L | B6-C | Lutein | 39.94 | |
| | | Neoxanthin | 11.61 | 0.22 |
| | | Violaxanthin | 1.55 | |
| E139L | B5-C | Lutein | 45.13 | |
| | | Neoxanthin | 17.33 | 0.26 |
| | | Violaxanthin | 2.78 | |
| E139L/R142L | B5-C | Lutein | 33.5 | |
| | | Neoxanthin | 0.89 | 0.02 |
| | | Violaxanthin | 1.78 | |
| E180L/R70I | A1-A/B | Lutein | 50.97 | |
| | | Neoxanthin | 33.32 | 0.38 |
| | | Violaxanthin | 2.34 | |
| N183L | A2-A | Lutein | 51.53 | |
| | | Neoxanthin | 25.93 | 0.32 |
| | | Violaxanthin | 2.2 | |
| Q197L | A3-A | Lutein | 44.84 | |
| | | Neoxanthin | 26.23 | 0.34 |
| | | Violaxanthin | 5.41 | |
| H212V | B3-D | Lutein | 44.4 | |
| | | Neoxanthin | 21.77 | 0.31 |
| | | Violaxanthin | 4.25 | |

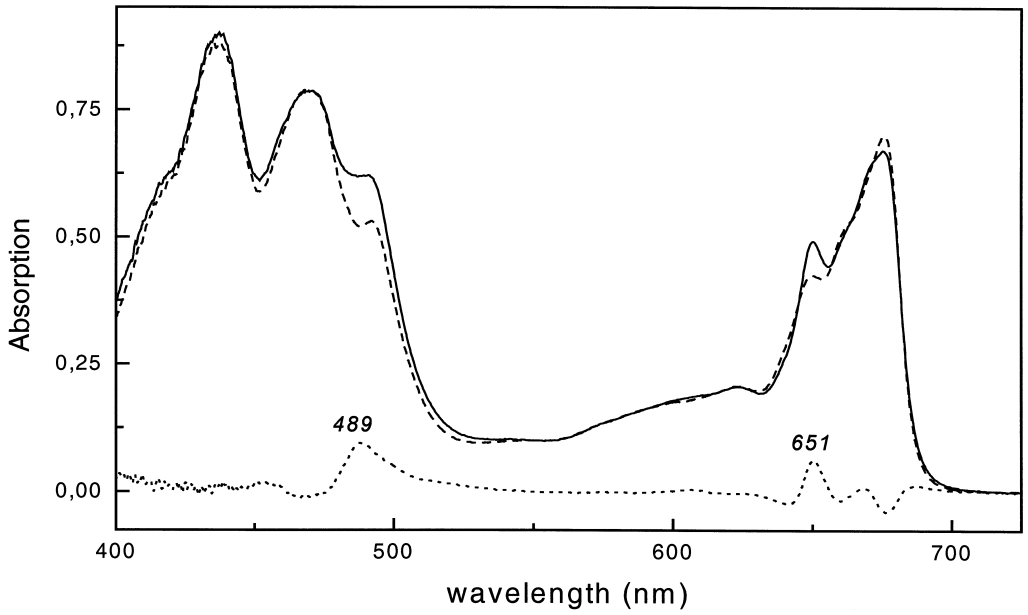


Fig. 1. Low temperature absorption spectra (100 K) of recombinant LHCII Lute+Neo (binding two lutein and one neoxanthin molecule per mol of polypeptide) (solid), and of LHCII Lute (binding two lutein molecules but not neoxanthin) (broken). The lower curve represents the difference spectrum upon normalisation to the same area in the Qy transition (630–700 nm).

LHCII in the presence of Chl *a* and Chl *b* plus either lutein as the only carotenoid or both lutein and neoxanthin. As previously reported, this procedure yields recombinant LHCII proteins with respectively two luteins bound or two luteins and one neoxanthin per polypeptide while the chlorophyll complement is seven Chl *a* and five Chl *b* in both cases [9]. The LHCII Lute and the LHCII Lute+Neo, thus obtained, only differ in the former having its neoxanthin site vacant.

In Fig. 1 the absorption spectra at 100 K and their difference spectrum, upon normalisation at the same total absorption over the chlorophyll Qy transition region (630–700 nm), are shown. The major signal in the difference spectrum (Qy region) is detected at 651 nm due to the absence of the strong Chl *b*-neoxanthin interaction which is the cause of the typically prominent aspect of the Chl *b* absorption band in LHCII. This major component is only marginally sensitive to the coefficient used for the normalisation procedure (data not shown). At lower energies only minor differences were observed, most probably due to a shift of a Chl *a* absorption form from 669 to 677 nm.

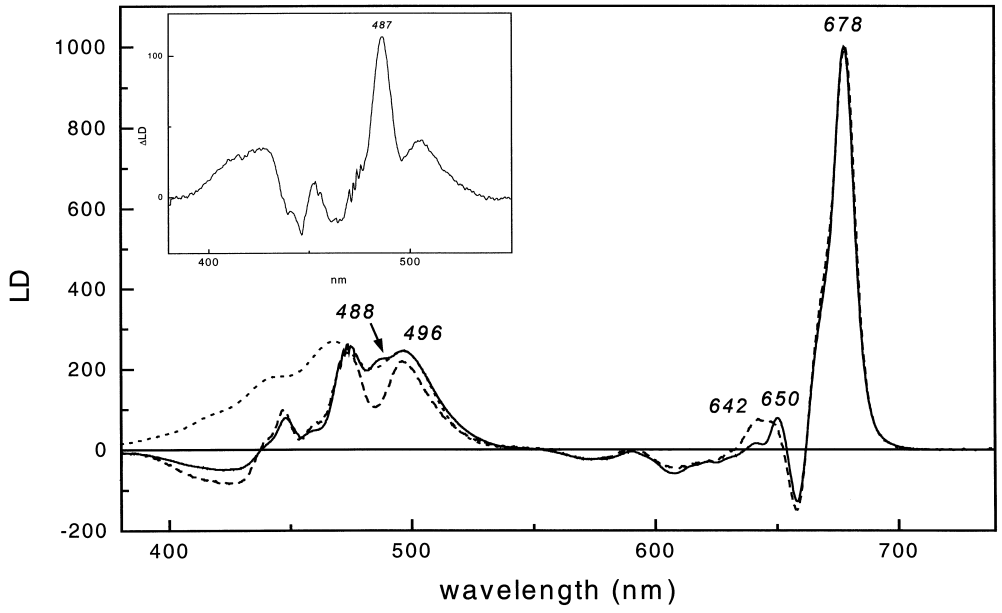


Fig. 2. Linear dichroism spectra (100 K) of LHCII Lute+Neo (solid) and of LHCII Lute (broken). The spectra were normalised to the same amplitude at the Chl *a* redmost transition (678 nm). The dotted line represents the spectrum of purified lutein in 80% acetone shifted by 20 nm to the red in order to fit the 496 nm signal of LD spectrum. See text for further information. Inset: LD difference spectra (solid minus broken) showing the LD signal of neoxanthin in LHCII. Arrow, neoxanthin signal in the LHCII Lute+Neo LD spectrum.

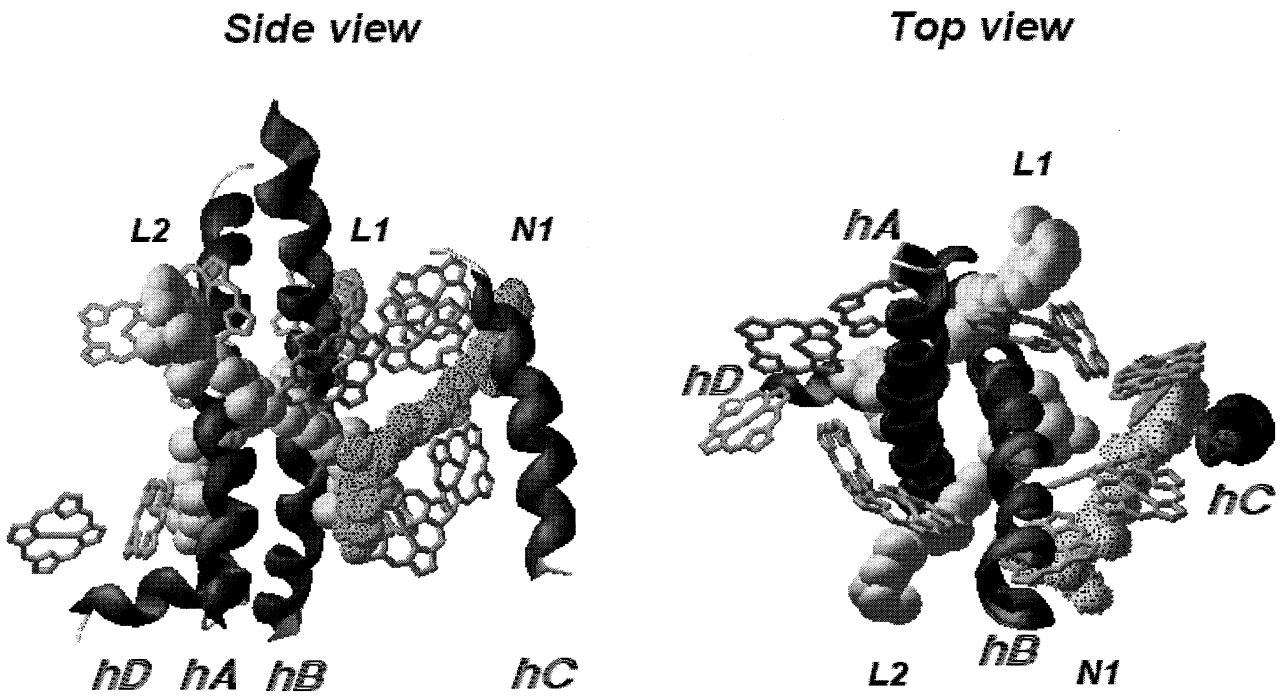


Fig. 3. Molecular model showing the localisation of neoxanthin in the LHCII structure as deduced from the present study. Neoxanthin is dotted to stress the fact that its localisation within the LHCII structure is inferred from biochemical and spectroscopic data, while lutein organisation was determined by electron crystallography [3]. L1, lutein site 1; L2, lutein site 2; N1, neoxanthin site; hA,B,C,D, helix A,B,C,D. Co-ordinates of the protein structure were kindly supplied by W. Kuhlbrandt (MPI-Frankfurt).

Xanthophylls absorb in the Soret region where a positive signal in the difference spectrum is observed at 489 nm. This feature is attributed to the redmost absorption band of neoxanthin according to previous results [9]. Neoxanthin also has two bands at higher energy but these do not appear in the difference spectrum due to the increased Chl *b* extinction in the Soret band caused by its interaction with neoxanthin [9].

The LHCII Lute and LHCII Lute+Neo proteins described above were analysed by linear dichroism (LD) spectroscopy. In Fig. 2 the LD spectra are shown upon normalisation to the same amplitude at the major, Chl *a*, peak. This signal was used for normalisation because only minor differences were observed between the absorption spectra of these two samples in this region [9]. Accordingly, the LD spectra were essentially identical at wavelengths above 658 nm while differences were observed between 630 and 658 nm indicating that interaction with neoxanthin may induce changes in the orientation and/or absorption of Chl *b* molecules. The amplitude of the LD signal depends on the angle $\phi$ between the dipole transition moment of the chromophore and the normal to the plane in which the protein is oriented. It should therefore be possible to estimate the orientation of the neoxanthin in LHCII by comparing the LHCII Lute to the LHCII Lute+Neo samples.

Our approach to this aim consists in using the two lutein molecules, whose orientation with respect to the LHCII structure is available from electron crystallography [3], as internal reference for the calculation of the unknown orientation of neoxanthin within the same protein structure. In order to perform the calculation it is thus necessary to identify the signals of lutein and neoxanthin in LD spectra and their contributions to the amplitude of the signal. In the blue region, a 488 nm signal, only observed in the neoxanthin containing protein, could readily be attributed to neoxanthin. The redmost absorption band of the lutein in the LHCII complex peaks at 495 nm [9,26]. A 496 nm signal, present in both LHCII Lute and LHCII Lute+Neo, was instead assigned to the redmost absorption band of lutein since chlorophyll absorption in this spectral range is very low while lutein is the only other chromophore.

The three absorption bands of carotenoids in the blue region are due to different vibrational states of the same (S2) electronic state rather than to different transitions; it could therefore be expected that a set of three peaks would show up in the LD spectra with similar amplitude rather than the single one observed (Fig. 1). This is probably due to overlapping of the two xanthophyll absorption bands at higher energy and the positive and negative signals of Chl *a* and Chl *b* absorption at wavelengths lower than 480 nm thus complicating the interpretation. In the following we therefore rely only on the redmost xanthophyll band ($>480$ nm) which is not hindered by Chl absorption.

The amplitude of the lutein contribution to the LD spectrum was obtained by sizing the absorption spectrum of purified lutein, shifted by 19 nm to the red to reproduce the absorption in LHCII [9,26], in order to fit the LD spectrum (Fig. 2). This procedure closely reproduced the 480–540 nm region of the LHCII spectrum. The total area of this lutein spectrum was considered to be the spectral contribution of the two luteins in sites L1 and L2 [9] of the LHCII structure [3]. It is thus possible to calculate the orientation of their dipole moment with respect to the normal to the plane on which the protein is oriented by using the co-ordinates of the structural model [3]. The dipole moment of the lutein, lying on the polyene chain, forms angles of 56.4° and 59.4° respectively for

sites L1 and L2 with the crystallographic $z$ axis corresponding to the normal to the membrane plane.

The LD signal is given by:

$$LD = 3/2\ A\ (1-3\cos^2\phi). \quad (1)$$

By using LD = area of the lutein contribution to the LD spectrum (arbitrary units); $A = C \times$ extinction of lutein (255 $cm^{-1}$ $\mu g^{-1}$ ml) [27], where $C$ is a normalisation factor; $\phi$L1 = 56.4; $\phi$L2 = 59.4, in Eq. 1, the $C$ factor was obtained, correlating the angle $\phi$ to the amplitude of the LD signal.

The contribution of the neoxanthin molecule to the LD spectrum was then obtained from the difference spectrum between LHCII Lute+Neo and LHCII Lute (Fig. 2, inset) showing a major peak at 487 nm which corresponded well to the neoxanthin absorption. As previously described for lutein, the absorption spectrum of neoxanthin in acetone was shifted by 20 nm to reproduce the absorption in LHCII [9] and sized to fit the difference spectrum. The total area of this spectrum was used as the value of the contribution of neoxanthin to the LHCII LD spectrum. This value was 2.52 times lower than the corresponding value for the two luteins in sites L1 and L2.

By using Eq. 1 with LD = area of the neoxanthin contribution to LD spectrum (lutein L1+L2 contribution/2.5); $A = C \times$ extinction of neoxanthin (223 $cm^{-1}$ $\mu g^{-1}$ ml); with $C$ as determined above for lutein, a $\phi$ angle value of 57.6° for neoxanthin in LHCII was determined by this procedure.

## 4. Discussion

Out of three xanthophyll molecules tightly bound to LHCII, two are located in the helix A/helix B domain, which constitutes the twofold symmetrical core of this pigment-protein, as revealed by structural analysis [3], while the third one is bound to a still unknown site. In vitro reconstitution with different carotenoids has shown that two sites bind lutein and/or violaxanthin while neoxanthin is restricted to the third site [9]. The occupancy of the former two sites provides stabilisation of LHCII folding while the latter site can remain vacant without affecting this function. Spectroscopic analysis provided evidence that lutein binding sites are in close contact with Chl *a* chromophores, which were found to be associated with the helix A/helix B cross in the homologous protein CP29 [24], while the neoxanthin site was associated with Chl *b* chromophores located more peripherally near either helix C or D [9,24]. These data strongly suggest that lutein is bound to the L1 and L2 sites resolved in the structural model [3]. In this work we investigated the location of the neoxanthin binding site by studying the effect of mutating amino acid residues, coordinating single chlorophylls to different domains of the protein, to non-binding residues. Only mutations on helix C resulted in changes of the LHCII xanthophyll composition, thus decreasing neoxanthin content. This strongly suggests that the neoxanthin site is associated with the helix C domain, the most probable location being in the space between helix C and the helix A/helix B cross where the chlorophylls co-ordinated to helix C residues 131, 139 and 142 protrude [3]. Several chlorophyll sites are packed in this protein domain consistent with the strong Chl *b*/neoxanthin interaction observed by absorption and circular dichroism spectroscopy [9]. Actually, linear dichroism analysis (Fig. 2) shows that the presence or absence of neoxanthin induces changes in the orientation of Chl *b* chromophores. Together with the absence of obvious xanthophyll binding motifs in the lumenal and stromal loops connecting helix C, this might suggest that neoxanthin is held in place by interactions with chlorophylls. It is worth noticing that reconstitution in the absence of neoxanthin yields a LHCII protein showing major changes on Chl *b* while Chl *a* is very little affected implying that Chl *b* is preferentially bound by helix C with respect to Chl *a*. Additional information on the organisation of neoxanthin within LHCII was obtained by using linear dichroism spectroscopy which provides information on the orientation of the chromophore transition moment with respect to the normal to the plane on which the proteins are oriented by the polyacrylamide squeezing method. During sample preparation for LD, the detergent concentration is decreased below the critical micelle concentration (c.m.c.) thus inducing membrane proteins to aggregate into platelets by their hydrophobic surfaces, originally embedded in the lipid membrane, as shown by electron microscopic analysis [27]. The normal to the orientation plane can thus be considered to correspond to the crystallographic twofold axis in the LHCII structural model [3]. In order to perform calculation of the neoxanthin $\phi$ angle between the transition moment of the xanthophyll and the normal to the orientation plane the two lutein molecules for which the orientation is known [3] were used as internal reference and a normalisation $C$ factor was obtained correlating the $\phi$ value to the amplitude of the lutein contribution to the LD spectrum. This normalisation factor was then used to obtain the $\phi$ value for the single neoxanthin in LHCII from its contribution to the LD spectrum which was readily obtained from the difference of LD spectra of the two samples having the neoxanthin site either occupied or vacant. Whereas some error in the calculations might be due to approximation in the fitting procedure, it is likely to be small due to the following considerations: (i) $\phi$ angle values below 55° can be excluded, which would yield a negative LD signal around 488 nm; (ii) while a contribution of Chl *b* to the 488 nm signal used for calculations might be present, it has to be rather small since Chl *b* Soret absorption was shown to peak at 465 nm [9], thus sufficiently apart; (iii) the amplitude of the neoxanthin LD signal is comparable to those of the lutein molecules, resulting in a similar orientation. Calculations assuming $\phi$ angle values above 58° result in LD untenably high compared to the experimental spectrum. We estimate the orientation to be correct within a range of ± 1.5°. The above conclusions rely on the determination of the absorption contributions of lutein and neoxanthin to the absorption and LD spectra. While it is not clear if the extinction of xanthophylls in LHCII is different with respect to that in organic solvent, the similar values for the red shift of the S2 transition induced by their binding to LHCII suggests the environment is rather similar for lutein and neoxanthin [9]. Thus, while we cannot exclude changes in the extinction of xanthophylls upon binding to LHCII, these should similarly affect both xanthophylls and therefore should not affect the accuracy of our estimation to a significant extent.

On the basis of the results of point mutation analysis and of polarisation spectroscopy we propose an organisation of neoxanthin within the LHCII structure as shown in Fig. 3. It can be noticed that the orientation of neoxanthin with respect to the normal to the membrane plane is similar to that of luteins suggesting this is due to constraints originating from the mol-

ecule length and the contrasting hydrophobicity of the polyene chain and of the end rings owing to the presence of -OH and epoxide substituents. LD cannot distinguish between two symmetrical orientations of neoxanthin with respect to the normal axis. We favour the one shown in Fig. 3 with the epsilon ring directed towards the stromal end in close contact with helix B on the basis of preliminary results showing that mutations on Gly-78 make neoxanthin binding weaker (M. Gastaldello and R. Bassi, unpublished results).

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