The light harvesting complex (LHCIIb) of photosystem II can be reconstituted in vitro from its recombinant apoprotein in the presence of a mixture of carotenoids and chlorophylls a and b. By varying the chlorophyll a/b ratio in the reconstitution mixture, the relative amounts of chlorophyll a and chlorophyll b bound to LHCIIb can be changed. We have analyzed the chlorophyll stoichiometry in recombinant wild type and mutant LHCIIb reconstituted at different chlorophyll a/b ratios in order to assess relative affinities of the chlorophyll-binding sites. This approach reveals five sites that exclusively bind chlorophyll b. Another site exhibits a slight preference of chlorophyll b over chlorophyll a. The remaining six sites are filled preferentially with chlorophyll a but also tolerate chlorophyll b when this is offered at a large excess. Three of these chlorophyll a-affine sites could be assigned to distinct positions defined by the three-dimensional LHCIIb structure. Exclusive chlorophyll b sites complemented by chlorophyll a sites that are selective only to a certain extent are consistent with the observation that chlorophyll b but not chlorophyll a is essential for reconstituting stable LHCIIb. These data offer an explanation why a rather constant chlorophyll a/b ratio is observed in native LHCIIb despite the apparent promiscuity of some binding sites.

Chl a/b-binding proteins contain regions of high homology (1, 2), but their assembled pigment-protein complexes can nevertheless be distinguished by their pigment stoichiometry. LHCIIb binds 7 Chl a, 5 Chl b, two luteins, and one neoxanthin besides violaxanthin, which appears in substoichiometric amounts unless special care is taken during solubilization (3). This stoichiometry holds true for LHCIIbs isolated so far from a wide range of higher plants (1, 4, 5), indicating a high selectivity of binding sites for either Chl a or Chl b. On the other hand, the successful reconstitution of recombinant LHCIIb in the absence of Chl a suggests that at least some of the Chl-binding sites exhibit little or no selectivity (6). Under extreme depletion of Chl a in the reconstitution mix (Chl a/b = 0.01) complexes with a Chl a/b ratio of 0.03 could be isolated (7). Spectroscopic and stoichiometric data indicated that under these conditions Chl b molecules are bound into Chl a sites. But the selective enrichment of Chl a in the emerging complexes also points to at least one binding site that shows quite pronounced affinity for Chl a.

This selectivity might be based on the recognition of the relatively small structural difference between a methyl versus a formyl group (Chl a versus Chl b, respectively). The introduction of electronegative groups into tetrapyrroles has an impact on the basicity of the nitrogenous coordinating the central magnesium atom (8). The withdrawal of electrons from the pyrrole nitrogens by the formyl group in Chl b renders the central magnesium a stronger Lewis acid which would be required for stable interaction with weaker Lewis bases (9). So both the strength of a ligand selecting more or less pronounced Lewis acidity of the central magnesium as well as interactions with formyl or methyl groups of Chls b and a, respectively, have to be considered as possible molecular tools tuning the selectivity toward either Chl. However, structural features that contribute to selective binding of Chls a or b by non-polar or polar interactions with the methyl or formyl group are not yet known. At the present crystallographic resolution of 3.4 Å, obtained with two-dimensional crystals of LHCIIb, Chls a and b cannot be distinguished nor is it possible to fit their phytol chains into the electron density map, therefore, the orientation of the Chl molecules is unknown (10). Moreover, the interaction of the formyl or methyl groups of Chl b or a, respectively, with their protein environment which is likely to contribute to the a/b specificity of Chl binding, could not be identified.

The crystallographic study did, however, describe magnesium ligands for 9 of 12 Chls bound to LHCIIb (10). Referring to these binding sites, several groups (11–13) chose a mutagenesis approach to elucidate further the positions of Chls a and b within LHCIIb. Single amino acids were exchanged, and these point mutants of light-harvesting Chl a/b-binding protein were analyzed for their Chl composition and content after reconstitution. Although some of these mutants turned out to lose more
than one Chl molecule and/or more than one type of Chl, the various studies led to largely similar Chl assignments, confirming most of the initially deduced ones. Chls a1, a2, and a5 (numbering of Chls as in Ref. 10) were unanimously identified as Chl a and Chls b5 and b6 also were confirmed. Chls a3, a4, and b3 either were interpreted as mixed binding sites or the obtained results did not coincide. The remaining Chls are more difficult to address because they could not be assigned to a specific amino acid and therefore could not be easily eliminated by site-specific mutagenesis. In the case of Chl d6, a carboxyl-coordinated binding type, only Remelli et al. (11) suggested a mixed occupancy with Chl a and Chl b, whereas Rogl and Kühlbrandt (12) chose a mutation that severely affected both complex stability and trimerization capability, making a clear assignment more difficult. Chls a7, b1, and b2 were only addressed indirectly by comparing pigment stoichiometries and spectroscopic data of other mutants losing more than one Chl (11). Also taking into account Chl-Chl distances, the authors suggested Chl a7 as Chl b, Chlb1 as Chl a but confirmed Chlb2. In conclusion, these in vitro studies unanimously describe specific binding sites for Chl a (4 sites) and Chl b (4 sites). The remaining sites were either identified as binding either Chl or were not assigned at all.

In order to specify further Chl binding in LHCIIb, we undertook a systematic study of Chl a/b selectivity of binding sites in recombinant LHCIIb. We set out to perform a series of reconstitution assays at a wide range of Chl a/Chl b ratios and then analyzed the composition of pigments actually bound by the reconstituted complexes. Relative affinities toward Chl a and Chl b of binding sites were calculated from measured titration curves, following the previously described model applied for the determination of xanthophyll binding affinities in LHCIIb (14). Seven Chl-binding site mutants were used to confirm the binding affinities determined with wild type LHCIIb. Three mutant proteins were allowed to assign relative Chl a/b specificities to individual binding sites of LHCIIb.

EXPERIMENTAL PROCEDURES

Reconstitution and Purification of Pigment-Protein Complexes—Isolation and purification of overexpressed Lhcb1*2 (15) or mutants thereof were done as described before (16). Pigments were isolated from pea and purified by reversed-phase chromatography (C18, Waters Associates, Eschborn, Germany) using an acetone/water gradient (76–100% for Chl a; 88–100% for Chls b). Reconstitution (detergent-exchange procedure) and purification of assembled complexes (partially denaturing PAGEs) were performed as described previously (17). Extraction of pigments from purified complexes and quantification were done as described previously (14).

Chlorophyll-binding Site Mutants—Lhcb1*2 mutants used in this study are named according to the position and nature of the amino acid exchange. E65A, H68A, E139A, E180A, N183A, and H212A were described previously (12), and Q197E was described previously (13).

Fitting of Titration Curves—Relative affinities of 12 binding sites toward Chl a and Chl b were determined on the basis of an algorithm presented previously (14). In short, the Chl a/b ratio within a particular binding site is determined by the product of the relative affinity (Ka/b = hchla/hchlb) in this site and the Chl a/b ratio offered during complex formation. After normalization of bound ratios to one Chl per binding site the overall ratio in reconstituted LHCIIb at a given Chl a/b ratio can be calculated as shown in Equation 1.

![Fig. 1. Partially denaturing PAGE of reconstitution assays.](Image)

By varying 12 relative K_a/b values, measured titration curves were fitted in an iterative procedure arriving at a minimum value for the sum of the root of squared relative deviations at single data points.

In addition to the iterative fitting, a genetic algorithm was used to identify K_a/b values. Parameters for the genetic algorithm are as follows: 30 individuals each carrying 12 genes representing K_a/b values for 12 Chl-binding sites which were set to random numbers in the first cycle. 50% of the population, which is selected according to their fitness, undergoes a reproductive phase, yielding the initial population size. The offspring population contains individuals with changed combinations of genes because of the possibility of recombination events. The cycle is then completed by selection of the fittest individuals. In order to prevent the algorithm from being captured at local optima, a mutation rate of 0.2 was applied, and mere selection of the fittest individuals was avoided by exchanging 5 randomly chosen individuals of the selected population with 5 randomly chosen individuals of the remaining population.

A maximum of 20,000 cycles was set.

Additionally the results of the manual fitting procedure and the genetic algorithm were challenged by a more systematic screening of a large number of possible combinations of K_a/b values. Due to limited processing capacity we restricted this calculation to the following ranges with step widths given in parentheses: site 1, 1–50 (1); sites 2–6, 1–20 (1); site 7, 0–2 (0.1); and sites 8–12 were fixed at a value of 0.

RESULTS

Monomeric LHCIIb can be refolded from its denatured apoprotein Lhcb1 in the presence of Chls and xanthophylls. In this study, we used the detergent exchange method to reconstitute LHCIIb. Lhcb1 and pigments are dissolved in a mixture of the anionic detergent dodecyl sulfate and the non-ionic detergent octyl-β-D-glucopyranoside. Precipitation of dodecyl sulfate as its potassium salt then prompts protein folding and pigment binding. This is a rather mild procedure, allowing the isolation of reconstituent complexes even when they have a lower stability than native LHCIIb. In order to titrate Chl a/b-binding sites, we performed a series of reconstitutions with either Chl a or Chl b or various mixtures of these two, whereas all other components such as xanthophylls stayed the same. The resulting pigment-protein complexes were then isolated on a partially denaturing polyacrylamide gel. Fig. 1 shows that Chl b alone, in combination with xanthophylls, is sufficient for LHCIIb reconstitution, whereas Chl a is not. The excess of Chl over protein was chosen such that over the entire range of Chl a/b ratios enough of either Chl was present to fill all 12 binding sites. Even so, at an offered ratio of Chl a/b = 9.2 a marked reduction of the reconstitution yield is observed, whereas Chl a/b mixtures between 0.1 and 3.2 (2nd to 6th lanes) result in roughly the same reconstitution efficiencies as a total pigment extract of thylakoids (10th lane).

Fig. 2 gives the relative pigment stoichiometries in LHCIIb reconstituted at varying Chl a/b ratios. Lutein and neoxanthin appear at a virtually invariable stoichiometry of 2:1, respectively, independently of the applied Chl mixture. By contrast, the contents of Chls a and b within the successfully refolded complexes are strongly affected by the offered ratio of these two pigments in the reconstitution mixture. When pigment contents are normalized to three carotenoids, these largely varied
Chlorophyll a/b ratios are required to obtain a complete picture of relative binding affinities, we used an algorithm that was presented in a previous publication (14), describing relative binding affinities of three xanthophyll pigments in LHCIIB. The composition of Chl a and Chl b in any Chl-binding site can be calculated as the product of the Chl a/b ratio in the mixture and the ratio of binding affinities for either Chl. After normalization of relative ratios to one Chl per each filled site, the total numbers of Chl a and Chl b per monomeric LHCIIB can be calculated. Thus, by varying 12 independent relative affinities toward Chl a and Chl b, the measured pigment compositions can be fitted. This model calculation assumes each of 12 positions being occupied with Chl and does not take into account any potential cooperative effects.

Fitting of titration curves was done by three methods. One approach was to vary 12 affinity values \((K_{ab})\) by hand, monitoring the effect of the variations in a simple table calculation. The minimum sum of relative deviations of calculated Chl a/b ratios as compared with measured data (mean of 13 independent competition series) at each point was taken as an indicator for optimal fitting. As a control a genetic algorithm was employed with a population of individuals each carrying a set of 12 independent traits which correspond to the affinity values and started at random values. For both methods one cannot completely exclude the possibility of the iteration being captured at local minima. By means of a permutative enumeration within a large range of values for every single site, we tested the values as derived from both methods. All three methods lead to a nearly identical distribution of relative affinity values. Because fitting was done with 12 variables but 6 competition points, we do not expect to precisely define 12 different values. However, the fitting procedures defined three categories of Chl-binding sites in LHCIIB (Fig. 3).

A first set is composed of 5 sites that exclusively bind Chl b \((K_{ab} = 0)\). In another position \((K_{ab} = 0.6)\) Chl b binds 1.7 times better than Chl a, making this the only member of a Chl b affine but not an exclusive class of binding sites. Finally 6 Chl a-preferring sites representing relative affinity values of 5 to 6 were identified. These classes of affinity values yield an almost perfect fit of calculated values (solid line) with measured data points (Fig. 3). Due to the aforementioned restrictions because of the limited number of data points, we tested the significance of the three classes of binding sites with regard to both the number and strength of their relative affinity values.

**Chl b-Only Sites**—As is already obvious from Fig. 2, the Chl b content does not fall below 5 molecules per monomeric complex even at a high Chl a/b ratio offered during refolding. This is reflected by 5 values of \(K_{ab} = 0\). Probing this number with deliberately changed values such that the calculated titration curve stays within the margins of standard deviation at high Chl a/b ratios results in significantly worse fits of data. Changing a Chl b-specific site into a site strongly preferring Chl b \((K_{ab} = 0.6)\) from 0 to 0.035) results in a significant increase of Chl a binding in the model compared with measured values at higher Chl a/b ratios offered (see column A and dashed line in Fig. 3), leading to a 3-fold increased sum of relative deviations. Vice versa, the Chl b affine site \((K_{ab} = 0.6)\) can only be transformed into a more selective site for Chl b but not into a Chl b-specific site as becomes obvious when decreasing the affinity by a factor of 3 \((K_{ab} = 0.2\), column B and dotted line in Fig. 3). Consequently, the number of five exclusive Chl b-binding sites is significant.

**Chl a- and Chl b-Affine Sites**—According to Fig. 3 LHCIIB
contains a group of six Chl a-prefering sites that exhibit quite uniform relative affinities toward Chls a and b. Considering the determination of 12 values of $K_{ab}$ on the basis of 6 data points, we have to question the significance of both the number of positions representing one class of binding sites and the effect of a coupled decrease and increase of values within one group. Table I depicts the impact of changing the $K_{ab}$ values within the Chl a-prefering class of binding sites. Inserting one (columns B–D) or two (columns E and F) sites with enhanced Chl a affinity leads to a concomitant decrease of the $K_{ab}$ for the other members of Chl a-prefering sites. This also has an impact on the site with rather weakly expressed Chl b affinity.

To compensate for an overall reduction of the slope, this site is turned into a nonspecific or slightly Chl a prefering site. However, when the value of $K_{ab} = 0.6$ is also fixed, the sum of relative deviations is only slightly increased (columns C and F).

Taken together, these data clearly define 5 binding sites that are exclusively filled with Chl b, one site being rather unspecific toward Chl a and Chl b, and 6 sites that more or less preferentially but not exclusively bind Chl a. These data do not allow an unequivocal determination of $K_{ab}$ values as far as selective but not specific sites are concerned. The varied $K_{ab}$ values shown in Table I more or less spoil the accuracy of fitting; however, these effects certainly are not strong enough to justify a final determination. In order to overcome this restriction, we also analyzed a number of Chl-binding site mutants with respect to their binding selectivity toward Chl a or Chl b.

All mutants tested in this study exhibited constant carotenoid stoichiometries over the whole range of Chl a/b ratios (data not shown), so again we considered the Chls bound per 3 carotenoids as the Chls bound per monomeric LHCCIb. By comparing the number of bound Chls in wild type and mutant LHCCIb at various Chl a/b ratios, we determined how many Chls were lost due to the mutation. From all suggested combinations of $K_{ab}$ values in Table I (columns A–F), every possible affinity value or, in case of 2 Chls lost, every combination thereof was omitted. Resulting calculated titration curves were validated according to their sums of relative deviation. So every mutant gives a minimum sum of relative deviations for a given combination of $K_{ab}$ values. The average of these sums for combination C from Table I is 0.27, whereas columns A, B, and D–F give worse fits with averages of 0.31, 0.29, 0.3, 0.32, and 0.46, respectively. Therefore, Chl a/b binding characteristics of the analyzed mutant proteins suggest the presence of at least one Chl-binding site with pronounced affinity toward Chl a.

What are the $K_{ab}$ values lost by the mutations and how well do the remaining sites match the measured titration curves? Table II summarizes the values that were omitted in order to minimize the deviations. The resulting pigment compositions are compared with measured data in Fig. 4. Similarly to wild type LHCCIb (see above) some mutants also bind less Chl at low Chl a/b ratios. Because calculations were done with integral numbers of Chls (10, 11, or 12) over the entire range of the competition, we observed a slight deviation of calculated numbers especially at low Chl a/b ratios. By comparing solid lines of calculated values with measured data points, it becomes obvious that this effect is caused mainly by Chl b. This indicates that, due to its excess at very low Chl a/b ratios, Chl b is bound into slightly Chl a-prefering sites but then is lost during purification (see “Discussion”).

The $K_{ab}$ values of four mutants were calculated on the basis of having lost 2 Chls (E65A, H68A, Q197E, and H212A) with respect to wild type titration. Whereas this value holds true for E65A and H212A over the whole range of competition, H68A does not show the extra loss of Chl at Chl b excess. By contrast, Q197E loses nearly three Chls at both Chl a and Chl b excess. All these mutants lose one Chl a affine site ($K_{ab} = 4–5$). Mutants E65A, H68A, and H212A also lose the moderately Chl b affine site ($K_{ab} = 0.6$), whereas fitting of Q197E titration curve is in its optimum when a Chl b-only site is omitted from calculation.

Whereas the total number of Chls remains unchanged in mutant E139A, we clearly observed an exchange of Chl a with Chl b. This effect manifests itself in the middle region of the competition, which is consistent with the transformation of a slightly Chl a-prefering site into a Chl b-prefering site ($K_{ab} = 5$ into $K_{ab} = 0.1$).

Mutant E180A loses Chl b below an offered Chl a/b ratio of about 0.5. Above this ratio Chl a accounts for the overall loss of one Chl. This behavior suggests the mutation of a Chl a affine site ($K_{ab} = 4$) which also accepts Chl b when offered in excess. By contrast, Chl b binding in mutant N183A is virtually the same as in wild type LHCCIb, whereas Chl a is lost regardless of the offered Chl a/b ratio. In this case the mutation seems to have hit a more pronounced Chl a affinity ($K_{ab} = 20$).

**DISCUSSION**

In this work, we present the first systematic assessment of relative Chl a/b specificities of Chl-binding sites in LHCCIb by titrating these sites with Chl a and Chl b. We found three different classes of Chl-binding sites by measuring the Chl a/b ratios of the recombinant complexes in dependence on the Chl a/b stoichiometry offered in the reconstitution mixture; 5 sites bind Chl b virtually exclusively, whereas one binding site only shows a slight preference for Chl b. The remaining 6 binding sites prefer Chl a to Chl b but are not Chl a-specific. A preference of either Chl a or Chl b means that the other Chl can be

**Table I**

**Effect of $K_{ab}$ variations in binding sites 1–7**

| Binding site | A | B | C | D | E | F |
|--------------|---|---|---|---|---|---|
| 1            | 6 | 20| 20| 50| 20| 20|
| 2            | 6 | 5 | 5 | 5 | 5 | 20|
| 3            | 5 | 4 | 4 | 4 | 3 | 3 |
| 4            | 5 | 4 | 4 | 3 | 3 | 3 |
| 5            | 5 | 3 | 3 | 2 | 2 | 2 |
| 6            | 6 | 1 | 0 | 0 | 0 | 0 |
| 7            | 10.7 | 13.2 | 17.6 |
| SRD          | 4.6 | 6.8 | 9.6 | 10.7 | 13.2 | 17.6 |

**Table II**

**Localization of binding affinities**

| Wild type | Chls | Chls | Chls | Chls | Chls | Chls |
|-----------|------|------|------|------|------|------|
|           | 1–100 | 1100 | 1100 | 1100 | 1100 | 1100 |
| 6–50      | 20   | 5–20 | 20   | 20   | 20   |
| 3–6       | 5    | 5    | 5    | 5    | 5    |
| 3–5       | 4    | 4    | 4    | 4    | 4    |
| 3–5       | 4    | 4    | 4    | 4    | 4    |
| 3–5       | 4    | 4    | 4    | 4    | 4    |
| 3–5       | 4    | 4    | 4    | 4    | 4    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
| 0–6–2     | 0    | 0    | 0    | 0    | 0    |
bound if the preferred Chl species is minor to the other one in the reconstitution mixture. It should be noted that at all titration points, *i.e.* even at Chl \(a/b\) ratios of about 0.1 or 10, the minority Chl was abundant enough in the reconstitution mixture that it could have occupied all Chl-binding sites.

All stoichiometries of LHCIIb-bound Chls are calculated per 3 xanthophylls. In principle, stoichiometries could also be expressed as per protein molecule; however, the xanthophyll concentration in isolated recombinant LHCIIb can be measured significantly more precisely than the protein concentration (12, 13). The assumption that all recombinant LHCIIb contain \(3\) xanthophyll molecules is justified (i) by the high preference of two carotenoid-binding sites for lutein and one for neoxanthin (14), and (ii) by the observation of a virtually unchanged lutein:neoxanthin:violaxanthin stoichiometry of 2:1:0.1 throughout our titrations for both wild type (Fig. 2) and mutants (not shown).

For several binding sites we find a heterogeneous occupation with either Chl \(a\) or Chl \(b\); otherwise, the range of Chl \(a/b\) ratios observed in the recombinant complex at a constant number of Chl molecules per complex (Fig. 2) would not be possible. It should be noted that although a low stringency isolation method for recombinant LHCIIb was chosen in this work, the procedure is still stringent enough to warrant that all Chl molecules are bound to proper Chl-binding sites rather than interacting non-specifically with the protein. This is indicated by a consistent stoichiometry of 12 Chls per complex decreasing only very slightly at low Chl \(a/b\) ratios in the reconstitution mixture (Fig. 2, values for first three competition points are 11.3, 11.4, and 11.9 Chls per 3 carotenoids, S.D. = 0.67, \(n = 13\)). This is in good correspondence with results obtained under conditions of extreme Chl \(a\) depletion (offered Chl \(a/b\) ratio = 0.01) and after trimerization of monomeric complexes (7). Calculating these data on the basis of 3 carotenoids also gives 11 Chls with Chl \(a\) present in every third complex. What is the reason for the loss of one Chl in reconstitution experiments carried out at low Chl \(a/b\) ratios? It should be kept in mind that two binding sites exhibiting the same relative Chl \(a/b\) affinities need not necessarily bind their Chls with equal stability. Thus, we assume that the decreased Chl content at low Chl \(a/b\) ratios is due to one Chl \(a\)-preferring binding site where the binding of Chl \(b\) is rather unstable. Because this feature seems to be weakly expressed or lost in as many as three mutant proteins (H68A, E180A, and N183A), we currently cannot identify the responsible Chl-binding site.

The optimum fit of wild type data (Table I) implies a number of six binding sites exhibiting an equivalent and moderate preference of Chl \(a\) to Chl \(b\) (\(K_{a/b} = 5\) to 6). However, analysis of mutant apoproteins suggested the presence of at least one Chl-binding site with a pronounced affinity toward Chl \(a\) (\(K_{a/b} = 20\), see Table II). This was assigned to Chlo2 which is bound at Asn-183 and was shown earlier to be responsible for a long wavelength absorption band at 676 nm (18). In parallel with the 676-nm absorption signal, the N183A mutant also exhibits a loss of long wavelength fluorescence (12), making Chlo2 a good candidate for the function of energy transfer toward the
reaction center. So occupation of binding site a2 with Chl a would be particularly important for the proper function of LHCFb, consistent with our observation that a2 has the highest preference for Chl a of all binding sites. In a former study refolded complexes strongly enriched Chl a with respect to the offered ratio (Chl a/b = 0.03 versus an offered ratio of 0.01), yielding complexes that show two absorption features in the Chl a region (670 and 676 nm) (7). The occurrence of two spectral forms in this region under refolding conditions where there was just enough Chl a present to cover one binding site in each LHCFb monomer suggests that at least two sites are of pronounced Chl a affinity. However, LHCFb refolded in complete absence of Chl a also exhibits an absorbance feature between 665 and 680 nm (6), indicating that the 670-nm signal in Chl a-depleted LHCFb (7) actually might arise from a long wavelength form of Chl b. Thus, all these data are consistent with the notion of Asn-183 forming a high affinity Chl a-binding site giving rise to the 676-nm signal.

E139A is the only mutant in this study that does not lose Chl but rather exchanges Chl a with Chl b. Both at high and low Chl a/b ratios this exchange diminishes. If the amino acid exchange decreased a moderately expressed Chl a affinity, we would indeed expect a minor extent of this exchange at small Chl a/b ratios because at Chl b excess this site would predominantly be filled with Chl b anyway. The best fit of E139A titration curve was obtained when a K_{ab} value of 5 was replaced by 0.08 (see calculated lines in Fig. 4). Again, at an offered ratio of 9.23, this relative affinity implies a Chl a/b value of 0.74 in this site, which corresponds to 0.425 Chl a and 0.575 Chl b. The resulting difference as compared with the wild type (−0.6 Chl a and +0.6 Chl b) is also observed in the experimental data (Fig. 4). Chl-binding site Glu-139 has been addressed before both in LHCFb (11, 12) and the corresponding Glu-174 in CP29 (19). Upon exchanging Glu-139 by Ala or Leu, the loss of two Chls (about one Chl a and one Chl b) was observed (11, 12). However, both studies also describe the loss of about 50% of neoxanthin which clearly was not the case in the present work. This indicates that the reconstitution procedures and/or purification methods in those studies were more stringent than the ones in this work. The combination of time resolved absorption and dichroic spectra was the base of an alternative approach (20) questioning the Chl assignment of the crystallographic structure (10). In agreement with the present study the authors concluded from theoretical modeling that Chl5 (Glu-139) should actually be a Chl a (20). Also in the minor antenna complex CP29 position Chl5 was found to be occupied by 0.6 Chl a and 0.4 Chl b (19) at an offered ratio of Chl a/b = 8. Although this would correspond to a K_{ab} of 0.2, it can be concluded that in CP29 this site does not exclusively bind Chl b. Finally glutamate as a Chl ligating amino acid not only in this study showed selectivity toward Chl a. In a reciprocal amino acid exchange study it was shown that replacing Glu-131 with Glu in LHCFb increased the Chl a content, whereas the corresponding inverse exchange in CP29 (E166Q) decreased the Chl a/b ratio of refolded complexes (19). This also follows the notion that stable ligation to a weaker Lewis base (Gln as compared with Glu) demands the presence of a stronger Lewis acid such as Chl b as compared with Chl a (8). Based on the evidence of the present work together with corroborating observations in the literature, we suggest Glu-139 (Chl/b) to be a Chl a-prefering binding site.

Chl-binding site Glu-180 was shown to lose one Chl throughout the competition. Because the nature of the lost Chl obviously depends on the Chl a/b ratio offered for refolding, this site cannot be of pronounced affinity for either Chl a or b. Indeed, optimum fitting results in the assignment of a K_{ab} value of 4. This is in agreement with both former studies of mutant LHCFb (11, 12) and the initial assignment of Kühlerbrandt et al. (10).

The proposed assignment of positions Glu-139, Glu-180, and Asn-183 as Chl a sites questions the Lewis base strength of an amino acid as the sole determinant of Chl binding specificity. In particular the presence of the relatively weak Lewis base Asn (position 183) in a high affinity Chl a site suggests that in some cases other structural features must participate in the expression of Chl binding specificity. A hydrophobic pocket being able to harbor the Chl a-specific methyl group but providing an energetically unfavorable environment for the polar formyl group of Chl b may serve such a demand. Unfortunately this possibility is hard to address by site-specific mutagenetic approaches because the exact three-dimensional arrangements of most amino acid side chains in LHCFb are not known yet. However, provided the negative charge of Asp in this position does not interfere with complex formation, altogether it would be interesting to see whether this exchange will even enhance Chl affinity at this site.

Whereas mutants E139A, E180A, and N183A could be assigned to only one K_{ab} value, the other mutants lose more than one Chl preventing a straightforward localization of their respective binding characteristics. Chl loss in mutant Q197E additionally shows a slight dependence on the offered Chl mixture. Both at the lowest and highest Chl a/b ratio tested, we observed a loss of 3 and 2.5 Chls, respectively, whereas at intermediate Chl a/b ratios the loss is around 2 Chls. Therefore the omission of only two affinity values for fitting of the titration curve may not properly model the titration points at extreme Chl a/b ratios. However, because the average Chl loss amounts to 2.2 ± 0.5, we used the number of 10 Chls bound to Q197E as an approximation for our calculations. The loss of two Chls by a single mutation may be a short distance effect, where the binding of both pigments at least partially is dependent on pigment-pigment interactions. In this case we would expect an equivalent effect of mutating binding sites of two closely neighbored Chls. Remelli et al. (11) calculated the edge-to-edge distances between Chls in LHCFb on the base of the crystallographic structure (10). Interestingly those mutations of the present study that led to the loss of two pigments fall into two pairs of rather closely neighbored Chls. Chlr4 and Chlr5 are separated by 6.4 Å, whereas the distance between Chln3

![Fig. 5. Chl a/b binding behavior in refolded minor and major antenna complexes. LHCFb data are from this study, and minor antennae data were taken from the following references: CP24 (29); CP26 (28); and CP29 (26, 27).](http://www.jbc.org/Downloaded from)
and Chlb3 is only 4.2 Å. For Chla3, Chlb3, and Chla4 these distances by far define the nearest neighbor. Only Chlb5 is also located quite close to Chlb5 (4.9 Å) and Chla7 (7.9 Å). Glu-65 (Chla4) and His-68 (Chlb5) carry all characteristics of an interdependent pair of Chl-binding sites. They both lose an affinity value of 0.6 and a slightly Chl a-prefering site (K_a/b = 4 and 5, respectively). If we assume these K_a/b values to be located at Chla4 and Chlb5, then Chls a3 and b3 (bound to Gln-197 and His-212, respectively) are left with a Chl a affine site and a Chl b-only site (K_a/b = 4–5 and 0). His-212 is a stronger Lewis base than Gln-197. Consequently, His-212 was suggested to serve as a ligand for Chl a-binding site and Gln-197 as a Chl b-binding site (9). By contrast, these sites were either found to be of mixed occupancy (11, 13) or even described as Chl a sites (12). We conclude that the dual loss of affinity values cannot at present be satisfactorily unraveled.

However, results of the present study together with former publications cast an interesting light on the nature of Chl b-only sites. The identification of Gln-131 (Chl b5) as a Chl a-binding site and the observation of His-212 (Chlb3) and Gln-197 (Chla3) being mixed binding (11) or even Chl a sites (12) decreases the number of Chl b-binding sites with known ligand as suggested on the basis of crystallographic data (10) to one location (Gln-131, Chlb6), which indeed was shown to bind Chl b (11). Candidates for the remaining four Chl b exclusive sites are Chlb6, Chla7, Chlb1, and Chlb2. Interestingly, of these sites only for Chlb6 the peptide carbonyl of Gly-78 was identified as the ligand for the central magnesium (10). Chls a7, b1, and b2 are located close to the luminal and stromal surfaces of LHCCIb and were suggested to be bound in a similar way by peptide carboxyls in the loop regions (10). Indeed several amino acids both in the stromal loop connecting helices C and A (21) and in the luminal loop² between helices B and C were identified as crucial components of LHCCIb stability. This indicates that these regions might supply weak Lewis bases whose interaction with a Chl crucially contributes to overall complex stability thereby selecting the stronger Lewis acid Chl b.

The presence of five exclusive Chl b sites within LHCCIb must render its assembly strongly dependent on the availability of Chl b. Indeed LHCCIb abundance has repeatedly been correlated with Chl composition present in the thylakoid membrane. The barley mutant Chlorina f2 is deficient in Chl b and, therefore, does not accumulate the LHCCIb apoproteins Lhcb1 and Lhcb2 (22, 23). Consistently, Lhcb1 fails to reconstitute in the absence of Chl b (Fig. 1). Also plants grown in intermittent light (IML) accumulate only small amounts of Chl b and show a strong reduction in the level of Lhcb1 and Lhcb2 (LHCCIb) and Lhcb6 (CP24) apoproteins (24, 25). Chl a/b ratio of IML maize thylakoids was 8.8 (24), a Chl composition where the yield of reconstituted LHCCIb (offered Chl a/b ratio of 9.3, Fig. 1) also decreases in the present reconstitution assays. However, at a Chl a/b ratio of 5, as was measured in the IML thylakoids of barley (25) with strongly reduced amounts of Lhcb1 and Lhcb2 at the beginning of the continuous illumination, we would not expect the yield of in vitro reconstituted LHCCIb to drop as significantly as described. This might be due to the fact that in vivo Chl a/b-binding proteins, at least at an early stage of greening, compete for the limiting amount of Chl b present. The minor Chl a/b proteins CP29 (27), CP26 (28), and CP24 (29) have also been reconstituted at different Chl a/b ratios. These data are compared with the Chl binding behavior of LHCCIb in Fig. 5. The corresponding shape of the titration plot of LHCCIb and CP24 matches the observation that it is Lhcb1, Lhcb2, and Lhcb6 that are most strongly reduced in IML thylakoids under conditions of Chl b depletion. On the other hand CP26 and CP29 show a higher flexibility with respect to Chl composition, a feature that may be the reason for their preferential appearance under conditions of Chl b depletion. According to Marquardt and Bassi (24) complexes present at an early greening stage when little Chl b has been accumulated also exhibit higher Chl a/b ratios. CP24 and LHCCIb both bind 5 molecules of Chl b per apoprotein, whereas CP26 and CP29 carry 3 and 2 Chl b, respectively. By mutational analysis of Chl binding in CP29 four mixed binding sites were identified (19). CP29 was shown to refold in the complete absence of Chl b (26), although complexes were rather unstable. However, CP26 failed to reconstitute in the presence of only Chl a and xanthophylls (28), indicating that the additional binding site might correspond to one of the exclusive Chl b-binding sites described in the present work for LHCCIb.

Are those Chl-binding sites in LHCCIb that exhibit a low preference for either Chl a or Chl b in our titration experiments more selective in vitro? LHCCIb preparations from various green plants show an apparently quite constant Chl a/b ratio of around 1.4 (4, 5, 10), suggesting the existence of 7–8 Chl a and 5–6 Chl b-binding sites. Even so, it is principally possible that in vitro, a number of Chl-binding sites may contain a mixture of Chl a and Chl b. The overall Chl a/b stoichiometry in LHCCIb would then be kept constant by some unknown mechanism controlling the stoichiometry of Chl a and Chl b during LHCCIb assembly. Alternatively, if there is no mixed binding of Chl a and Chl b to individual binding sites in vitro, complexes with “correctly” filled Chl-binding sites may be selected for by their higher stability which would require finely tuned selection, distinguishing fully stable from slightly less stable complexes. It should be noted that the classification of Chl-binding sites according to their Chl a/b specificity as proposed here suggests a simpler mechanism of achieving constant Chl a/b ratios in assembled LHCCIb. Our titration studies revealed 5 Chl-binding sites that only accept Chl b; at the same time we have seen in reconstitution experiments that binding of Chl b is essential for stabilizing LHCCIb. We propose that during greening LHCCIb only starts to accumulate when enough Chl b is available to fill the 5 Chl b-exclusive binding sites. The other binding sites that all can accommodate either Chl a or Chl b then are filled with Chl a, as this is the more abundant Chl and thus is more readily available. Without any further requirements of specificity or control this will result in the observed Chl a/b ratio of about 1.4.

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