Chlorophylls, ligands and assembly of light-harvesting complexes in chloroplasts

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Abstract Chlorophyll (Chl) b serves an essential function in accumulation of light-harvesting complexes (LHCs) in plants. In this article, this role of Chl b is explored by considering the properties of Chls and the ligands with which they interact in the complexes. The overall properties of the Chls, not only their spectral features, are altered as consequences of chemical modifications on the periphery of the molecules. Important modifications are introduction of oxygen atoms at specific locations and reduction or desaturation of sidechains. These modifications influence formation of coordination bonds by which the central Mg atom, the Lewis acid, of Chl molecules interacts with amino acid sidechains, as the Lewis base, in proteins. Chl a is a versatile Lewis acid and interacts principally with imidazole groups but also with sidechain amides and water. The 7-formyl group on Chl b withdraws electron density toward the periphery of the molecule and consequently the positive Mg is less shielded by the molecular electron cloud than in Chl a. Chl b thus tends to form electrostatic bonds with Lewis bases with a fixed dipole, such as water and, in particular, peptide backbone carbonyl groups. The coordination bonds are enhanced by H-bonds between the protein and the 7-formyl group. These additional strong interactions with Chl b are necessary to achieve assembly of stable LHCs.

Keywords Chlorophyll · Chlorophyll b · Chlorophyll c · Chlorophyll molecular axes · Chlorophyllide a oxygenase · Coordination bonds · Dipole moments · Lewis acid · Lewis base · Ligands · Light-harvesting complex · Chloroplast development

Abbreviations
BChl Bacteriochlorophyll
CAO Chlorophyllide a oxygenase
Chl Chlorophyll
Chlide Chlorophyllide
D Debye
LHC Light-harvesting complex
LHCP Light-harvesting complex apoproteins
MCD Magnetic circular dichroism
Phlide Protochlorophyllide
PS Photosystem

Introduction
The dramatic developmental transformation performed by the chloroplast has attracted broad interest over the past several decades (see Hoober and Argyroudi-Akoyunoglou 2004; Wise and Hoober 2006, for reviews). Although the organelle displays a variety of features among different organisms, as revealed by electron microscopy, its monophyletic origin by endosymbiosis of an ancient cyanobacterium has received increasingly strong support (Palmer 2003; Bhattacharya and Medlin 2004; Rodriguez-Ezpeleta et al. 2005). Descendants of the primary endosymbiotic event branched into the glaucophytes, green algae, and plants, which contain chlorophyll (Chl) a and Chl b, and the red algae, which contain only Chl a. Tomitani et al.
phyt...dination of sidechains and introduction of oxygen atoms at specific locations. Oxygen is the most electronegative atom commonly found in biological systems and exerts significant effects on the electronic distribution in the Chl molecule.

We previously (Hooper and Eggink 1999; Eggink et al. 2001, 2004; Chen et al. 2005) proposed that modifications at the periphery of the Chl molecule influence the coordination chemistry of the central Mg atom and that this effect plays an important, if not major, role in the interaction of Chl b with LHC apoproteins (LHCPs) and thus in the assembly of LHCs. In particular, we proposed that, as a Lewis acid, the Mg atom in Chl b favors axial coordination bonds with harder Lewis bases than does Chl a. This proposal was supported experimentally by direct measurement of equilibrium constants of various tetrapyrrole derivatives with specific ligands (Tamiaki et al. 1998). In an unbiased chemical context, Chls a and b should then prefer different ligands. Recent evidence demonstrated, however, that Chl a interacts with a broad range of ligands, from the imidazole group of histidine to water. Chl b, on the other hand, is found only with ligands containing an oxygen atom.

Chlorophylls a and d

Conversion of 3,8-divinyl-Chl a to 3-monovinyl-Chl a by reduction of the 8-vinyl group to an ethyl group is the final step in Chl a biosynthesis and yields the predominant form of Chl a (Nagata et al. 2005). As a result, Chl a has electron-donating methyl and ethyl groups at positions 7 and 8, respectively (Fig. 1). Along with reduction of the C17–C18 double bond to a single bond, which converts the porphyrin precursor protochlorophyllide (Pchlide) to the chlorin ring system, these groups impose an electron density, from opposite sides of the molecule along the X axis, on the pyrrole nitrogens, which partially shields the positive charge of the central Mg atom. In addition, the 3-vinyl and 13^1^-keto groups exert weak electron withdrawing effects on opposite ends of the Y axis.

The geometrical coordinates of the molecular framework for Chl a are shown in Fig. 1. The primary X axis transects the molecule from the position of C17 to C7. The Y axis transects the molecule from C2 to C12.

Summary of differences in chlorophylls

The spectral differences between the species of Chls in chloroplasts expand the ability of photosynthetic organisms to harvest light. However, the spectral range of Chl a alone is broadened by various micro-environments within complexes with proteins (Nishigaki et al. 2001; Croce et al. 2002; Linnanto et al. 2006), which argues against spectral differences per se as the raison d’être for occurrence of the “secondary” Chls, in particular, Chls b and c. Overall properties, not only their spectral features, are altered as consequences of chemical modifications on the periphery of the molecules. Important modifications are desaturation of sidechains and introduction of oxygen atoms at specific locations. Oxygen is the most electronegative atom commonly found in biological systems and exerts significant effects on the electronic distribution in the Chl molecule.
The experimentally determined, functional $Q_y$ transition-moment direction is a vector 70° clockwise from the X axis (Fragata et al. 1988; Simonetto et al. 1999; Sundholm 2003; Cai et al. 2006). This displacement from the geometrical Y axis places the functional $Q_y$ vector from near C1 to near C11. The functional $Q_x$ vector is within a few degrees of the geometrical X axis.

Chl is synthesized by oxidation of the 3-vinyl group to a formyl group, whose electron-withdrawing character further extends the $Q_y$ vector but should also cause the transition direction to align more closely with the molecular Y axis. As a result, the lowest energy absorption band shifts from 665 nm (in methanol) for Chl $a$ to a longer wavelength (lower energy) maximum of 697 nm for Chl $d$ and increases the dipole strength, which is proportional to the molar absorption coefficient. The transition dipole is strengthened further in BChl $a$, which has an electron-withdrawing acetyl group on C3 and a single bond between C7 and C8. The $Q_y$ absorption maximum is shifted to 772 nm (in methanol), with a dipole of 7.2 Debye (D) (at a refractive index for the environment of 1.35) (Knox and Spring 2003). The electronic distribution in BChl $a$ is more symmetrically aligned along the Y axis, with an elliptical electron density, and the $Q_y$ transition-moment direction is essentially perpendicular to the X axis, i.e., from C2 to C12 (Sundholm 2003).

Chlorophyll $b$

Synthesis of Chl $b$ involves incorporation of the electronegative oxygen atom to generate the 7-formyl group, which, as an aromatic aldehyde, is expected to have a dipole moment for the group of approximately 3.0 D (Desyatnyk et al. 2005). The oxygen provides a significant pull on electrons away from the core of the molecule along the X axis, which weakens the $Q_y$ dipole strength of the molecule from 5.33 D for Chl $a$ to 4.41 D in Chl $b$ (at a refractive index for the environment of 1.35) (Knox and Spring 2003). Since the dipole strength determines the magnitude of the absorption coefficients, among other properties, the absorption coefficient of Chl $b$ is only 50–62% (depending on solvent) of the $Q_y$ absorption peak of Chl $a$ and is shifted to higher energy, with a maximum at 652 nm (in methanol). The $Q_y$ transition-moment direction is displaced further from the Y axis than in Chl $a$ and is at an angle of only 61° clockwise from the X axis (Simonetto et al. 1999), described as a transect from near C20 to near C10.

Intuitively, the $Q_x$ transition moment of Chl $b$ should be stronger than that of Chl $a$. Computational analysis of molecular orbitals suggest that the $Q_x$ oscillator strength of Chl $b$ is 2-fold greater than for Chl $a$, with a maximum at 538 nm (Linnanto and Korppi-Tommola 2004). The
magnetic circular dichroism (MCD) spectrum of Chl \(b\) contains a weak negative transition at 540 nm and an intense negative transition at 625 nm (Frackowiak et al. 1987). From the argument that a stronger molecular dipole is consistent with a longer wavelength absorption maximum, the longer-wavelength negative transition in the MCD spectrum of Chl \(b\) may correspond to the \(Q_x\) transition.

The molecular orbital calculations suggest that, with weaker \(Q_y\) and stronger \(Q_x\) transitions, the electronic distribution in the Chl \(b\) molecule is essentially circular (Hoff and Amesz 1991). The lessened electron density around the pyrrole nitrogen atoms lowers the \(pK\) values for these atoms by about two pH units (Phillips 1963; Smith KM 1975). The withdrawal of electron density from the pyrrole pyrrole nitrogens results in less shielding of the Mg atom of Chl \(b\) and allows it to more strongly express its positive point charge. Molecular orbital calculations give the Mg in Chl an atomic charge with a value of +0.7 to +1.3 (Linnanto and Korppi-Tommola 2004).

**Chlorophyll \(c\)**

Chl \(c\) is found with Chl \(a\) in chromophyte algae, where it functions as a light-harvesting pigment. Whereas most of these algae do not contain Chl \(b\), some Prasinophycean algae, such as *Montioniella squamata*, accumulate a Chl \(c\), 3,8-divinyl-Pchlide, along with Chls \(a\) and \(b\) (Schmitt et al. 1994; Green and Durnford 1996). The red alga that contributed its plastid in the secondary endosymbiotic event apparently lacked Chl \(c\). Ability to make Chl \(c\) was the solution these organisms found to solve the problem that was accomplished in chlorophytes with Chl \(b\). As shown in Fig. 1, the structure of Chl \(c_3\) has several unique features. The \(c\)-type Chls characteristically retain the C17–C18 double bond that occurs in the porphyrin precursor, Pchlide \(a\). In addition, a trans double bond is introduced between C17\(^1\) and C17\(^2\) in the sidechain, which extends conjugation of the ring \(\pi\) system to the usually unesterified, electron-negative C17\(^3\) carboxyl group. Conversion of the propionate sidechain of Pchlide \(a\) to the acrylate sidechain of Chl \(c\) inhibits the ability of NADPH:Pchlide oxidoreductase to reduce the C17–C18 double bond as occurs in Chl \(a\) synthesis (Helfrich et al. 2003), which suggests that sidechain desaturation occurs prior to potential interaction of Pchlide \(a\) with the oxidoreductase. Thus Chl \(c\) retains the porphyrin ring system. The three major sub-types of Chl \(c\) (\(c_1\), \(c_2\) and \(c_3\)) occur as the result of oxidation of the C7 and/or C8 substituents. The C7 methyl group is modified to a methylcarboxylate (–COOCH\(_3\)) in Chl \(c_3\) and the C8 vinyl group remains unreduced (Porra 1997). These remarkable modifications all lie on the X axis of the molecule. Absorbance spectra of the series (\(c_1\), \(c_2\) and \(c_3\)) suggest that the functional \(Q_x\) transition moment increasingly dominates the long-wavelength absorption peaks (Jeffrey and Wright 1987; Helfrich et al. 2003), further reduces the absorption coefficient and blue-shifts the \(Q_y\) absorbance maximum to 630 nm for Chl \(c_1\) (in acetone). Additional modifications, such as esterification of the acrylate sidechain with galactosyl diacylglycerol, increase the number of minor forms of Chl \(c\) (Garrido et al. 2000).

A summary of ligands of chlorophylls in light-harvesting complexes

Coordination bonds are formed between Lewis acids and bases. A Lewis acid has an unfilled orbital that can accept a pair of electrons. A Lewis base (ligand) has a pair of unshared electrons that are available for donation to the Lewis acid to form a donor-acceptor complex. Lewis acids and bases are characterized as “soft” or “hard” according to their chemical properties (Jensen 1978). Soft species tend to bond by short-range orbital interactions, while hard species interact primarily by electrostatic forces.

The central Mg atom of Chl molecules, as the Lewis acid, interacts with proteins by formation of coordination bonds with an amino acid sidechain as the Lewis base. Compression of the electron cloud toward the Y axis of the Chl \(a\) molecule, as when the C17–C18 double-bond and C8 vinyl group are reduced, tends to shield the Mg atom and effectively reduces the electronegativity of the metal. This results in weaker interaction with the negative end of a fixed dipole or even repulsion of negatively charged groups. In contrast, Chl \(b\) Mg atom is less shielded and more strongly expresses its positive charge. Electron density in Chl \(b\) is also pulled outward by H-bonds between the 7-formyl group and other structures, which further enhances the \(Q_x\) transition moment. Substituting the central Mg in BCHl \(a\) with other metals (e.g., Ni) of greater electronegativity strongly influences the \(Q_y\) but not the \(Q_y\) transition energies of the tetrapyrrole molecule (Hartwich et al. 1998). In Chls \(a\) and \(b\) the metal is the same, but the argument can be applied in reverse, in which substitution of peripheral groups on the \(Q_y\) axis alters the environment of the central Mg ion and thus its effective electronegativity.

If H\(^+\) is considered as a Lewis acid, the availability of electrons in a Lewis base should be reflected in its \(pK\) value (Jensen 1978) (\(pK\) values vary dramatically from those obtained in aqueous media when the ionizable group is located in a nonpolar micro-environment (Mehler et al. 2002)). The electron pair available on an amine nitrogen atom binds H\(^+\) strongly (\(pK\) ~9). Although the nonpolar micro-environment within a protein molecule may lower the \(pK\) of an amino group of lysine (Gunner et al. 2000, Springer
ligands having a low pK where electrostatic interactions are more likely with Chl b. Thus Chl a interacts as expected with imidazole, its predominant partner, but also with amide sidechains and water. Chl b does not interact significantly with imidazole, whereas the peptide backbone carbonyl group and water are favorable ligands. Orbital interactions may play a greater role in coordination bonds with ligands containing accessible electron pairs (i.e., high pK values) such as imidazole, whereas electrostatic interactions are more likely with ligands having a low pK value such as carbonyl groups. This comparison suggests that interactions of the Chls with ligands involve more than simply the availability of nonbonded electrons on the ligand. In addition to differences in the properties of the Lewis bases, the more exposed positively charged Mg ion in Chl b is more electronegative and acts as a harder Lewis acid than the metal in Chl a. Thus Chl b should favor electrostatic bonds with groups containing a strong, fixed dipole.

With the exception of the imidazole group, ligands to Chl contain oxygen. Lewis bases that contain oxygen atoms are polar, with a “fixed” dipole, and tend to be “hard” Lewis bases. The structures listed in Table 1, which are common or potential ligands of Chl, are ordered according to increasing dipole moment of the monomeric molecule, as calculated by ab initio methods, to emphasize the importance of this parameter. When these molecules interact by H-bonding with other molecules, as in a solution or a crystal structure, the dipole moments increase or a crystal structure, the dipole moment is increased to a value of 4.80 D (crystal structure), with the predominant contribution to the dipole provided by the N(1)-H bond (Spackman 1992). In aqueous solution, the dipole moment is enhanced to a value of 3.96 D by H-bonding (Table 1). Both N atoms have a small negative charge, and the electron density is distributed nearly symmetrically (Fig. 2). The aromatic character of imidazole allows the dipole to reorganize in response to interaction with another structure.

His120, at the luminal end of helix-2 in LHCII (see Fig. 3), is not a ligand to Chl a, possibly because of its exposure to the thylakoid lumen where competition with water is greater than within the membrane. Also, the imidazole group may be protonated at the pH of the lumen during active photosynthesis, estimated to be near pH 5 (Kramer et al. 1999; Sacksteder et al. 2000). His212, a ligand to Chl a, is also near the luminal surface of the membrane but is likely shielded from the aqueous lumen by helix-4 of the LHC protein.

The imidazole group provides a good example of the attractive/repulsive forces that limit the strength of the
coordination bond. A repulsive force should exist between the \( \pi \) clouds of Chl and the ligand. However, the pair of electrons on N3 is an attractive force that satisfies the needs of the Lewis acid, Mg. The concept of electronegativity equalization (Noy et al. 2000) suggests that a partial charge (about 0.3e) is transferred from imidazole to the metal ion upon coordination (analyzed with Ni-BChl \( a \)). This shift in electron density should reduce the role of the dipole in the coordination bond and generate a partial positive charge on the ligand that is accommodated by the electron cloud of the conjugated \( \pi \) system of Chl \( a \). In contrast, the more exposed positive charge on Mg in Chl \( b \) likely repels the

| Ligand      | \( pK^a \) | Solution Dipole (D) | ab initio Dipole (D) |
|-------------|-----------|---------------------|----------------------|
| Carboxyl group |          |                     |                      |
| Formic acid   | 3–5       | 1.41 (University of Southern Maine website 2006) | 1.52 (Dudev et al. 1999) |
| Alcohol       | \( \sim -2 \) (Herschlag and Jencks 1989; Smith and March 2001) | 1.70 (University of Southern Maine website 2006) | 1.94 (Dudev et al. 1999) |
| Water         | \( -1.74 \) (Herschlag and Jencks 1989; Smith and March 2001) | 2.70 (Gregory et al. 1997) | 1.868 (Gregory et al. 1997) | 1.855 (Dyke and Muenter 1973) |
| Imidazole     | 6–7       | 3.96 (Spackman 1992) | 4.80 (Spackman 1992) | 3.66 (Spackman 1992) |
| Amide         |           |                     |                      |
| Formamide     |           | 3.84 (Spackman 1992) | 4.13 (Dudev et al. 1999) |
| Acetamide     | \( -0.62 \) (Grant et al. 1983) | 3.87 (Spackman 1992) | 3.69 (Spackman 1992) |
| Sidechain     |           | 3.46 (Antoine et al. 2002) | 4.2 (Gunner et al. 2000) |
| Peptide bond  |           |                     |                      |
| N-Methylacetamide | \( -0.42 \) (Grant et al. 1983) | 4.2 (Whitfield et al. 2006) | 3.73 (Whitfield et al. 2006) |
| Urea          | 0.053     | 5.15 (Abramov et al. 1999) | 4.56 (Spackman 1992) |
|              |           | 7.04 (Abramov et al. 1999) | 5.56 (Abramov et al. 1999) |

\( ^a pK^a \) for the conjugate acid reaction: \( AH^+ \leftrightarrow A^- + H^+ \)

\( ^b \) Crystal form

References are indicated in parenthesis

Fig. 2 (A) The structure of the imidazole group of histidine and (B) its electronic charge density, determined by X-ray diffraction at 103 K for the projection in (A) (adapted from Epstein et al. 1982). In (A), \( R \) = remainder of the histidine molecule.
positive charge that develops on the ligand. Thus the attractive force is limited by the repulsive force created between Mg and the induced positive charge on the ligand.

**Carboxyl group**

Two ligands for Chl \( a \) in LHCII are charge-compensated ion-pairs formed by electrostatic interaction between the sidechain carboxylate of glutamic acid and the guanidinium group of arginine. Although at pH 7 the carboxyl group has a negative charge and multiple pairs of unbonded electrons, these electrons are distributed between the two electronegative oxygen atoms in a resonance structure and are less available for protonation than in the imidazole group. This property is reflected in the higher-proton concentration (pH 3–5) required to protonate the carboxylate in solution. In proteins, its p\( K \) varies from a low of about 2, to a high of nearly 9 (Gunner et al. 2000; Georgescu et al. 2002; Laurents et al. 2003; Li et al. 2004). The higher p\( K \) values occur when the protonated carboxyl group is stabilized in a nonpolar environment (Mehler et al. 2002). The protonated carboxyl group has a relatively low ab initio dipole moment of 1.52 D (Table 1). When ionized, the negative charge is likely repelled by the \( \pi \) electron cloud of Chl \( a \). As the LHC apoprotein folds, the approach of a positively charged guanidinium group attracts electron density from the carboxylate group and the ion-pair is stabilized by the nonpolar environment. The resulting glutamate in this ion-pair is a soft Lewis base, with a dipole moment probably near that of a protonated carboxyl group.

The dipole moment of the ion-pair is possibly too low to displace a water molecule from Chl \( a \). With its electron cloud pulled away from the central Mg (\(+1\) charge), Chl \( b \) is expected to coordinate more readily with a carboxyl group (\(-1\) charge) and thus form a bond with largely electrostatic character. In LHCII, Chl \( b \) is coordinated with the sidechain of Glu139 near the stromal end of helix-2, which nevertheless is sufficiently near Arg142 for at least partial charge-compensation (Standfuss et al. 2005).

The ability of Chls to bind to imidazole and glutamate/arginine ion-pair ligands was assayed experimentally by interaction with a synthetic peptide that mimicked helix-1 in LHCPs. Chls \( a \) and \( d \) bound with nearly equal affinity, assayed by Förster resonance energy transfer from a tryptophan residue next to arginine (Chen et al. 2005). In contrast, Chls \( b \) and \( c \) did not interact significantly with the peptide. A theoretical analysis of the interaction of the Chls with the peptide (Chen and Cai 2007) strongly supports the experimental data and indicates that bonding of Chls \( b \) and \( c \) with these ligands is thermodynamically unfavorable (Table 2). In the presence of water, however, complex formation with Chl \( b \) is more favorable, which, as shown by molecular modeling, is the result of a water molecule bridging Chl \( b \) and the ligand (Chen and Cai 2007).

**Amide group**

The p\( K \) of an amide is \(-0.62\) (Grant et al. 1983), an indication that electrons on the oxygen or nitrogen are not readily available for bonding with H\(^+\). However, the group exhibits a relatively strong dipole, with the negative end on the oxygen atom. Sidechain amides in proteins have a dipole moment of 3.46 D (Table 1). The dipole moments of the model compounds, formamide and acetamide, are about 3.8 D in solution. The dipole is sufficiently strong to displace a coordinated water molecule from Chl \( a \) and...
complex more negative the value, the more thermodynamically stable the Chl b. Chl a

Species Heat of formation (kcal/mol)

|            | In vacuum | In water |
|------------|-----------|---------|
| Chl a      | −706      | −870    |
| Chl b      | 770       | −133    |
| Chl c₁     | 1,001     | 160     |
| Chl c₂     | 1,115     | 310     |
| Chl c₃     | 877       | 84      |
| Chl d      | −806      | −949    |
| BChl a     | −892      | −1047   |

The parametric method 5 was used to calculate the association of each of the species of Chl with a 16-mer maquette of helix-1 of Lhcb1 (Eggink and Hoober 2000) as described by Chen and Cai (2007). The more negative the value, the more thermodynamically stable the complex should also allow effective competition with water for Chl b within the environment of a membrane. In reconstituted complexes, several sites, including Gln197, have mixed occupancy (Bassi et al. 1999; Remelli et al. 1999), which suggests competition during folding. Yet the sidechain amide groups of Asn183 and Gln197 are ligands for only Chl a in LHCII in vivo, which suggests that Chl a is more abundant during folding and competes effectively by mass action.

Peptide bond

Backbone amides are usually H-bonded within helical or β-sheet structures in proteins and therefore unavailable for interaction with Chls. However, proline residues occur at conserved regions within Chl-binding proteins, particularly in the N-terminal region of LHCPS (Jansson 1999), which preclude formation of H-bonds to nearby backbone carbonyl groups. The pKₐ value for the model compound N-methylacetamide, an analog of the peptide bond, is −0.42 (Grant et al. 1983), slightly more basic than a sidechain amide group. The electron-donating methyl group bonded to the nitrogen provides a larger “pool” of electrons for the carbonyl oxygen to draw from, which is reflected in the slightly higher pKₐ value than of the sidechain amide group. This effect also increases the dipole moment of the carbonyl group. N-Methylacetamide has a calculated dipole of 3.73 D in the gas phase and a monomeric dipole moment of 4.2 D in liquid, which is the same as the calculated dipole moment of the peptide bond in proteins (Table 1). The ab initio dipole moment of the fully H-bonded N-methylacetamide in liquid is calculated to have an average of 6 D, with a spread from 4 to 8 D (Whitfield et al. 2006). Urea has an even stronger dipole moment than N-methylacetamide and also illustrates the effect of H-bonding. Its molecular dipole moment is 5.15 D and is increased to 7.04 D in the crystal form (Abramov et al. 1999). An electrophilic center such as the Mg atom of Chl should have a similar effect to that of H-bonding. As expected from the strong dipole moment of urea, the negative point charge on its carbonyl oxygen may be repulsed by the electron density enclosing the Mg of Chl a, which prevents it from being an effective ligand. Urea does not seem to compete with imidazole or the glutamate/arginine ion-pair, the most favorable ligands of Chl a (Eggink and Hoober 2000). However, it should effectively compete with other ligands for binding to Chl b.

A peptide bond carbonyl in association with a polarizing, positively charged Mg should have a dipole moment at least as large as H-bonded N-methylacetamide and therefore should form a strong electrostatic bond with Chl b. A free backbone carbonyl group in helix-1 in Lhcb1 occurs at Gly78, because of nearby Pro82, within the interior of the membrane. This group should displace water from a Chl molecule but is probably sterically hindered from coordinating directly with the Mg atom by the adjacent, bulky amino acid sidechains of leucine and phenylalanine (see Fig. 3). As a result, this ligand is bridged by a water molecule to Chl α6 in LHCI (Standfuss et al. 2005). That this position is occupied by Chl a rather than Chl b may be determined by the availability and order of binding of the Chls during assembly of the complex. Repulsion of the strong dipole by the electron cloud of Chl a possibly limits its direct interaction with backbone carbonyl groups.

Of particular interest is the finding that backbone carbonyls of proline residues provide ligands to Chl a in a water-soluble Chl-binding protein from Lepidium virgineum (Horigome et al. 2007). In the complex, four Chl α molecules are bound in a solvent-excluded pocket at the interfaces of the tetrameric protein. This observation is evidence that Chl a can fulfill the full range of ligand coordination with sufficient support from the local environment. However, this arrangement is quite different from the interaction of monomeric Chls with LHCPS during LHC assembly.

Phosphatidyl glycerol

An oxygen of the phosphodiester linkage in a phosphatidyl glycerol molecule serves as the ligand in LHCl to Chl a7, which resides near the stromal surface of the thylakoid membrane (Liu et al. 2004). This rare ligand is also found in photosystem (PS) I (Jordan et al. 2001). The side of the phosphodiester group opposite the Chl a molecule is H-bonded to sidechains of tyrosine and lysine residues (Liu et al. 2004), which probably reduces the electron density on the ligand oxygen. Approach of the negative end of a
strong dipole toward the Mg in Chl \(a\) should again be hindered by the electron density surrounding the metal.

**Implications for assembly of light-harvesting complexes**

The concept of ligand preference was developed largely because Chl \(b\) is found only in LHCs, at specific sites, and with rare exceptions is not found in core complexes. X-ray diffraction studies of crystallized reaction centers (Fromme et al. 2001; Jordan et al. 2001; Loll et al. 2005) and LHCCI (Liu et al. 2004; Standfuss et al. 2005) revealed unambiguous Chl-ligand pairs, without mixed occupancy. Whereas a nitrogen atom of the imidazole group of histidine is the most common ligand, sidechain amide groups, water molecules and even a few carboxylate groups occur as ligands of Chl \(a\) in reaction centers of PS I and PS II (Jordan et al. 2001; Balaban 2005; Balaban et al. 2002; Oba and Tamiaki 2002, 2005; Ferreira et al. 2004).

Although Chl \(b\) expresses more strongly a positive charge on the central Mg than Chl \(a\), and consequently interacts electrostatically more readily with hard Lewis bases, the above analysis indicates that Chl \(a\) can also bind to these ligands. However, a stable LHCCI cannot be reconstituted in vitro with only Chl \(a\). Conversely, stable LHCCI was reconstituted with only Chl \(b\), and the number of Chl \(b\) molecules (13.5) was the same as when both Chls were present (13.7) (Kleima et al. 1999; Reinsberg et al. 2001; Schmid et al. 2001). Thus, sites normally occupied by Chl \(a\) can be occupied by Chl \(b\). Whether Chl \(b\) molecules indeed interact directly with ligands of Chl \(a\) or are bridged by water molecules remains to be determined. As shown in Table 2, calculated \(\Delta H\) of formation of complexes with a synthetic peptide containing the glutamate/arginine ion-pair and a histidine residue indicated that complex formation with Chl \(b\) is thermodynamically unfavorable. However, insertion of a water molecule to bridge Chl \(b\) and the ligand dramatically increased thermodynamic stability (Chen and Cai 2007).

The overlap of ligand characteristics is substantial and too much may have been made about ligand selectivity with Chls. Therefore, unambiguous occupancy of specific binding sites as found in vivo must involve more than these properties. Two recent publications shed light on how the LHCCI complex is assembled in vivo. Reinbothe et al. (2006) showed that LHCPs are not imported at a detectable rate into plastids purified from a Chl \(b\)-less mutant of Arabidopsis thaliana. These authors also confirmed the localization of CAO on the inner membrane of the chloroplast envelope as reported by Eggink et al. (2004). An alternate site for CAO activity was achieved by Hirashima et al. (2006), who transformed the Chl \(b\)-less mutant of A. thaliana chl1-1 with the gene for CAO from the cyanobacterium Prochlorothrix hollandica to achieve active CAO on thylakoid membranes. In these plants, the higher Chl \(b\) content resulted in a Chl \(a/b\) ratio that approached 1, much lower than the ratio of 3–4 in wild-type plants. In LHCCI, the ratio was 0.8 in contrast to 1.3 in the complex from wild-type plants. Chl \(b\) was recovered in purified PSI and PSII core complexes, which normally lack Chl \(b\), as well as in LHCs. Because of the widespread distribution of Chl \(b\) in the transformed plants, Hirashima et al. (2006) concluded that the restrictive distribution of the Chls in wild-type plants is not the result of discriminatory binding affinities of Chl \(a\) and Chl \(b\) to ligands. Because the P. hollandica CAO on thylakoid membranes led to a widespread distribution of Chl \(b\), the conclusion emerges that active CAO only on the envelope of chloroplasts leads to restriction of Chl \(b\) to LHCs. To achieve incorporation of Chl \(b\) selectively into LHCs, as found in wild-type plants, assembly of LHCs should therefore occur during import at the level of the envelope.

In Chl \(b\)-less mutants of higher plants, only a few of the apoproteins for LHCI and LHCCI accumulate in the organelle in vivo (Król et al. 1995; Bossmann et al. 1997; Espineda et al. 1999). This observation has traditionally been interpreted as an indication that the proteins are rapidly degraded upon entry into the chloroplast stroma unless Chl \(b\) is present to allow stable integration into the thylakoid membrane. However, as noted above, chloroplasts from a Chl \(b\)-less mutant of A. thaliana lacked the ability to import LHCPs. Accumulation of LHCPs in vivo into the plastid of the alga Chlamydomonas reinhardtii was markedly reduced in the Chl \(b\)-less strain, cbn1-113 (Park and Hoober, 1997). Mature-sized proteins were detected in the cytosol, which indicated that import was aborted (White et al. 1996; Park and Hoober, 1997). In the absence of Chl, a condition achieved because the mutant strain was unable to synthesize Chl in the dark, accumulation of LHCPs in vivo was observed. Import and/or retention of LHCPs occurred in the organelle. Even in wild-type cells, excess LHCPs were shunted to vacuoles when the rate of Chl synthesis was insufficient to accommodate the rate of synthesis of the Chl-binding proteins (White et al. 1996).

**Model of LHCCI assembly**

Folding of a thylakoid membrane protein of cytosolic origin is a complex process, made more so by the environmental sensing of domains as the protein is threaded through the translocon in the chloroplast envelope. Popot
and Engelman (2000) and Bowie (2005) described two steps of the process of folding of a membrane protein. The first involves achieving the correct location and topology by the initially inserted segments. Second is the folding and condensation of the protein from this starting point. The evidence indicates that LHCP precursors achieve location and topology as they are guided by the transit sequence through translocons on the outer and inner envelope membranes. The transit sequence is removed soon after the N-terminal domain gains access to the stroma (Soll and Schleiff 2004; Vothknecht and Soll 2006). Membrane-spanning, nonpolar sequences that serve as stop-transfer domains are minimally 14–16 amino acids in length (Davis and Model 1985; Adams and Rose 1985; Popot and Engelmann 2000). In this respect, assembly of LHCs presents an interesting problem. Inspection of helix-1 of most LHCPs reveals that the length of the nonpolar sequence in the first membrane-spanning domain is only 10–12 amino acids long, which is on the short side of a significant stop-transfer signal. The nonpolar sequence is within the lagging half of the span, terminated by several charged amino acids (Green and Durnford 1996; Jansson 1999). Binding of Chl a to amino acids in the leading half of the helix, which is untypically polar and charged for a membrane-spanning segment of a protein (see Fig. 3), should increase the probability that this domain remains in the membrane.

It seems plausible to consider that as the N-terminal domain of LHCPs traverses the envelope inner membrane, sidechains of glutamate and arginine within the conserved sequence –EVIHSR– in helix-1 form a looped ion-pair ligand for Chl a. The histidine residue provides a second ligand for Chl a, as described by Eggink and Hoober (2000). Kohorn (1990) showed that mutation of this sequence, to replace histidine with alanine, eliminated the ability of the chloroplast to import a LHCP precursor. Binding of Chl b to these sidechains may allow this polar sequence to diffuse more readily into the nonpolar phase of the membrane. However, these interactions are insufficient to retain the protein in the membrane in the absence of Chl b. As shown by Chen et al. (2005), Chls b and c bind poorly to imidazole or glutamate/arginine ion pairs in vitro.

A possible ligand for the Chl b molecule that is necessary for retention of the protein in the plastid was suggested by the crystal structure of LHCII. Several backbone carbonyls near the N-terminus are precluded from H-bonding and formation of an $\alpha$-helix because of the richness of proline residues in this region of the LHCP. The carbonyl of tyrosine (Tyr24 in spinach Lhcb1) resides three positions distant in the amino acid sequence from a proline residue and is thus free to form a coordination bond with Chl b (Liu et al. 2004; Standfuss et al. 2005). The unusual abundance of proline in the N-terminal domain also extends to an iron-deficiency-induced (Tidi) protein, a homolog of the light-harvesting Chl $a/b$ proteins, in Dunaliella, which increases the probability of interaction with Chl b during this stress condition that leads to chlorosis (Varsano et al. 2006). The strong electrostatic bond formed by further polarization of the carbonyl dipole through interaction with Chl b may be essential to anchor a LHCP in the envelope membrane sufficiently long for the remainder of the protein to be transported from the cytosol to complete assembly (Fig. 4).

As the remainder of LHCP is transferred through the translocon, a second Chl b possibly coordinates with the backbone carbonyl of Val119 (Liu et al. 2004; Standfuss et al. 2005). Helices 2 and 3 are then transferred through the membrane, which would complete the first step described above by Bowie (2005). The rather weak hydrophobic character of helix-2, and the short nonpolar sequence (again the lagging half) in helix-3, suggest that these domains may enter the membrane largely unassisted, as found for other membrane proteins containing transmembrane sequences that are only moderately hydrophobic (Brambillasca et al. 2006). Other factors are also required at this step, probably to prevent helix-3 from escaping the membrane into the stroma (Fig. 4). One of these factors is the chloroplast signal-recognition particle. The ability of this complex to bind with high affinity to the loop between helices 2 and 3 (Tu et al. 2000) suggests that it plays an important role in this process (Schünenmann 2003). Other proteins such as Albino3 have been identified as important in the integration of LHCPs into the membrane (Moore et al. 2000; Bellafiore et al. 2002), although their specific actions are not known. Gerdes et al. (2006) found that Alb3 mutants were defective in chloroplast biogenesis but not in accumulation of LHCPs. With the overall disposition of the protein now achieved in the membrane, the glutamate/arginine ion-pairs between helices 1 and 3 can now form, which stabilizes the protein.

Although the $pK_a$ values vary widely, the magnitude of the dipole moments of the ligands that selectively coordinate with Chl a or Chl b are not substantially different, except for the backbone carbonyl group. Therefore, as Hirashima et al. (2006) conclude, highly specific ligand selectivity should not be expected. In vitro reconstitution of LHCs has been remarkably successful in reflecting the innate stability of Chl-ligand pairs (Bassi et al. 1999; Remelli et al. 1999; Rogl and Kühbrandt 1999; Horn and Paulsen 2004) but these experiments did not fully achieve the selectivity of interaction that is found in complexes that are assembled in vivo. Ligand selection can reasonably be considered by taking into account (i) the unique Lewis acid properties of Chl b, (ii) the preference of Chl b to form electrostatic bonds with hard ligands containing a fixed dipole (i.e., an oxygen atom), (iii) the micro-environments in which the interactions occur (i.e., the dielectric
constant), and (iv) the order of addition of the pigment molecules, which is determined partly by the local concentrations of the two Chls. Interaction of Chl b with sidechain amide groups would be expected from the properties of the ligands, and both Chl a and b are found with these ligands after in vitro reconstitution of LHCs (Remelli et al. 1999). Yet these groups are not ligands of Chl b in the crystal structure of LHII. Chl a is probably more abundant within the membrane and competes favorably for amide ligands by mass action during assembly. Thus, basing theoretical proposals for a specific Chl in each binding site on only the first three factors above is not sufficient. The lack of ambiguity, or mixed sites, found in complexes isolated from plants after in vivo assembly is most likely achieved also by the order in which Chls bind, determined by the relative concentrations of each.

An important aspect in the interaction of Chls and ligands is the location of the ligand within the protein structure. Tyr24 is near the stromal surface of LHII, in a region expected to have a membrane interface dielectric constant of 5–10 (Tanizaki and Feig 2005). As suggested below, association of Chl b9 with this residue may form prior to reaching this position in the membrane. Likewise, the carbonyl of Val119, non-H-bonded because of Pro116, is at the luminal end of helix-2 in LHII, also a region of relatively high dielectric constant. The backbone carbonyl group of Val119 coordinates with Chl b14. The Chl b molecules are possibly protected from the aqueous environment by the protein strand. H-Bonding of the 7-formyl group to Gln122 and Ser123 (Liu et al., 2004) would increase the Lewis acid strength of Chl b14 and strengthen this bond. These two Chl b molecules possibly stabilize an intermediate in the assembly pathway of LHII (Fig. 4).

Four Chl b molecules interact with helix-2 of LHcb1. Chl b12 forms a coordination bond with Glu139, which as discussed above is likely charge-compensated by Arg142. The other three Chl b molecules (b10, b11 and b13) retain water ligands (Fig. 3). H-Bonding of these Chl b molecules through the 7-formyl group would further enhance the electrostatic character of the Mg atom (Liu et al. 2004). The 7-formyl group of Chl b11 is H-bonded to the peptide bond N of Leu148, and along with Chl b12 may help to retain the stromal end of helix-2 in the membrane. The 7-formyl group of Chl b13 is H-bonded to the water ligand of Chl b10, which is in turn H-bonded via its 7-formyl group to the amide N of Gln131. These Chl b molecules, as an aggregate, may fill a void in the protein between helices 2 and 3 as the protein folds. Such an aggregate of Chl b molecules would be considerably more stable than a similar complex composed of Chl a. These molecules would then enter the structure late in assembly, as Horn and Paulsen (2004) and Horn et al. (2007) found during studies of the kinetics of reconstitution of the complex in vitro.

The argument is then reduced to one or two critical Chl b molecules that are required for retention of LHCPs in the chloroplast envelope during assembly—one that interacts with a backbone carbonyl near the N-terminus and possibly the second that binds to the luminal end of helix-2. Since the catalytic center of CAO is on the envelope inner membrane facing the intermembrane space (Reinbothe et al. 2006), the N-terminal domain of LHCP possibly binds to Chl b on that side of the membrane. As the protein is transported through the membrane, the bound Chl b would approach the stromal surface while the motif –ExxHxR– in helix-1 enters the interior of the membrane and binds Chl a (Fig. 4). These Chl b molecules may then hold the N-terminal domain in the inner membrane sufficiently long for the remainder of the protein to be

![Fig. 4 Model of LHII assembly in the chloroplast envelope and the proposed role of Chl b. Several proposed intermediates are shown in the sequence, left to right. After synthesis in the cytosol, a LHCP precursor is imported sufficiently into the chloroplast stroma for removal of the transit sequence from the N-terminus and for the first membrane-spanning region to engage the inner membrane. Chl a (dark green rectangles) binds to ligands in the motif provided by the ion-pair of the sidechains of glutamate and arginine and the imidazole group of histidine (dotted line, a). However, binding to these sites is not sufficient to retain the protein in the envelope. Without Chl b the protein slips back into the cytosol for transfer to vacuoles and subsequent degradation. Chl b (light green rectangles) forms a strong coordination bond with the peptide bond carbonyl of Tyr24, near the N-terminus, and provides an additional hold on the protein (solid line, a + b). Along with the Chl a molecules that bind to the motifs in membrane-spanning helix 1, Chl b binds to Try24 and the peptide carbonyl of Val119 at the luminal end of helix-2. These Chls retain the protein in the membrane sufficiently long for the remainder of the protein, including the conserved motif in membrane-spanning helix-3, to enter the membrane, bind additional Chl and xanthophylls molecules, and complete assembly (LHC). Other proteins in the membrane and stroma apparently assist assembly of the complete complex (see text).]
transported across the outer membrane of the envelope and become integrated into the inner membrane. This scenario ensures that Chl b enters the complex from the surface of the inner membrane that faces the outer membrane and thus occurs only in peripheral LHCs that are assembled with apoproteins synthesized in the cytosol.

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