In Vitro Reconstitution of a Light-Harvesting Gene Product: Deletion Mutagenesis and Analyses of Pigment Binding†

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ABSTRACT: AB96, a gene encoding a *Pisum sativum* chlorophyll *a*/*b* binding protein [Coruzzi et al. (1983) *J. Biol. Chem. 258*, 1399–1402], can be expressed in *Escherichia coli* and reconstituted with pigments by the procedure described by Plumley and Schmidt [(1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 146–150]. Following purification by polyacrylamide gel electrophoresis, the reconstituted pigment–protein complex (CP2) is shown to have similar pigment-binding characteristics to native CP2 complexes isolated from thylakoid membranes. Therefore, the AB96 gene product contains binding sites for chlorophylls *a* and *b* and xanthophylls, all of which are necessary for optimal reconstitution in vitro. Absorption, fluorescence, and circular dichroism spectroscopy indicate that the pigments are oriented accurately and that chlorophylls *a* and *b* are adjoined for energy transfer. Studies with proteins produced after deletion mutagenesis of AB96 indicate that NH_2-terminal amino acids 1–21 and COOH-terminal amino acids 219–228 do not play a role in pigment binding. In contrast, amino acids 50–57 and 204–212 (encompassing one of three conserved histidine residues) are essential for reconstitution. Residues near the presumed NH_2- and COOH-terminal α-helix boundaries (22–49 and 213–218, respectively) affect the stability of reconstituted CP2 during electrophoresis at 4 °C. Correlation of diminished chlorophyll *a* binding with disappearance of a negative circular dichroism near 684 nm suggests that amino acids 213–218 near the COOH-terminal boundary of the third membrane-spanning helix affect the binding of some chlorophyll *a* molecules.

Of the photosynthetic antenna complexes of higher plants, the chlorophyll *a*/*b* light-harvesting complex (LHCP II)[1] has been studied most intensely. Light-harvesting complexes are a class of chemically and functionally heterogeneous transmembrane pigment–protein complexes comprising about half of the thylakoid protein and pigment. Some are associated only with photosystem I (LHCP I) or photosystem II (LHCP II), but a fraction of LHCP II is mobile between the photosystems to spectrally balance excitation rates (Steinback et al., 1979; Allen et al., 1981). Recent reviews (Anderson, 1986; Anderson & Andersson, 1988; Chitnis & Thornber, 1988; Green, 1988; Green et al., 1991) summarize the state of the field.

A small nuclear multigene Cab family encodes the polypeptides of light-harvesting complexes (Dunsmuir, 1985; Pichersky et al., 1985; Chitnis & Thornber, 1988; Green et al., 1991). These proteins possess regions with high degrees of amino acid sequence similarity. It has been difficult to attribute the specific properties of pigment–protein complexes to individual gene products (Chitnis & Thornber, 1988; Green, 1988) because pigment–protein complexes purified from thylakoids contain a mixture of LHC proteins. Thus, it is not known how variations in primary structure of the pigment-binding proteins influence the structure, function, or regulation of antenna complexes. Biogenesis of the apoproteins involves precursor import across two chloroplast envelopes (Chitnis & Thornber, 1988; Keegstra et al., 1989), proteolytic processing to the mature size (Schmidt & Mishkind, 1986; Keegstra et al., 1989), energy-dependent insertion into the thylakoid membrane (Cline, 1986; Chitnis et al., 1987; Yalovsky et al., 1990), and assembly with pigments and other antenna complexes (Chitnis & Thornber, 1988; Green, 1988). Post-translational modifications, such as differential proteolytic processing (Kohorn et al., 1986), phosphorylation status (Allen et al., 1981; Anderson & Andersson, 1988), fatty acylation (Mattoo & Edelman, 1987), divalent cation binding [see Sibbald and Green (1987)], and binding of pigment, lipid, or detergent (necessary for purification from membranes), might also contribute to LHC heterogeneity (Green, 1988).

The composition of light-harvesting complexes depends to some extent on the detergent solubilization and isolation procedure utilized (Anderson & Andersson, 1988; Green, 1988), but the archetypal LHCP II appears to be organized as a trimer of M_r 25 000–30 000 polypeptide monomers with each monomer binding approximately 8 Chl *a* and 7 Chl *b* molecules (Butler & Kühlbrandt, 1988; Green, 1988). Xanthophylls are also integral components of LHCP II (Plumley & Schmidt, 1987), the stoichiometry per monomer being about 3 lutein and 1–2 molecules each of neoxanthin and violaxanthin (Braumann et al., 1982). In addition to their structural roles, the xanthophylls probably function in dynamic processes such as dissipation of excess excitation energy (Yamamoto, 1979; Demmig-Adams, 1990). Acyllipids do not appear to be integral components of monomeric LHCP II (Plumley & Schmidt, 1987), but galactolipids (Ikegami, 1982; Siefer-

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[1] Abbreviations: ACA, ϵ-aminocaproic acid; BAM, benzamidine; Cab, chlorophyll *a*/*b* binding; CD, circular dichroism; Chl, chlorophyll; CP-2, light-harvesting chlorophyll–protein complex of photosystem II following electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; IPTG, isopropyl thio-β-galactoside; LDS, lithium dodecyl sulfate; LDS–PAGE, lithium dodecyl sulfate–polyacrylamide gel electrophoresis at 4 °C; LHC, light-harvesting complex; LHCP I, light-harvesting complex of photosystem I; LHCP II, light-harvesting complex of photosystem II; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; TX100, Triton X-100.

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mann-Harms et al., 1987; Sprague, 1987) and phosphatidyldiacylglycerol (Maroc et al., 1987) appear to be important for functional energy transfer properties and for the formation of higher order aggregates which occur in vivo.

Much of the transmembrane LHCP II complex lies outside the membrane yet the pigments and polypeptides are inexplicably resistant to attack by protons and proteases/chemical reagents, respectively (Andersson et al., 1982; Siefermann-Harms & Ninnemann, 1982; Bürgi et al., 1987). Structural predictions of 3 transmembrane α-helices (Karlin-Neumann et al., 1985; Anderson & Andersson, 1988; Chitnis & Thornber, 1988) are supported by image analysis of two-dimensional crystals of LHCP II [Kühlbrandt & Wang, 1991; see also Evans and Nugent (1991)]. Two of the helices (A, B) contain more than 30 amino acids, including charged residues, and extend well beyond the membrane. Helix C is a more conventional transmembrane helix of about 20 amino acids. The stromal sides of the long helices are flanked by "hook" regions (Kühlbrandt & Wang, 1991) which may form from β-turns (Pichersky & Green, 1990). These regions are conserved among the various members of the LHC gene family of tomato (Pichersky & Green, 1990; Green et al., 1991) and are candidates for pigment-binding sites. Monomers associate into trimers possibly centered about a region near helix B (Kühlbrandt & Wang, 1991).

Spectroscopic evidence has provided insight into the organization of the light-harvesting pigments. Chlorophylls *a* and *b*, at least, are specifically oriented within their protein environment such that trimers of Chl *b* molecules with C_3 symmetry interact excitonically (Van Metter, 1977; Shepanski & Knox, 1981; Lin & Knox, 1988). Additional excitonic interactions between Chls *b* and *a*/*b* are apparent under certain conditions (Ide et al., 1987), but the organization of Chl *a* relative to that of Chl *b* is poorly understood. Crystallographic data suggest (Kühlbrandt & Wang, 1991) that chlorophylls are arranged into two layers parallel to the membrane with the porphyrin rings perpendicular to the membrane plane. Presumed interpigment distances (center–center) range from 9 to 14 Å, but the rings appear to be too distant from the polypeptide backbone for direct ligation of the central Mg atom by amino acid side chains. Instead, water molecules and H-bonding of the ring hydrogens may direct the pigment organization (Kühlbrandt & Wang, 1991). Although these results apply to an LHCP II complex which is a composite of different gene products, they are consistent with the occurrence of a limited number of residues which can serve as ligands for all 15 chlorophylls. The emerging picture for the structure and pigment-binding mechanisms of higher plant light-harvesting complexes differs radically from the situation for bacterial light-harvesting complexes and reaction centers. The bacterial pigment–proteins contain fewer pigment molecules per protein molecular mass, and the pigments are ligated by His, Gln, and Asn residues (Evans & Nugent, 1991; Green et al., 1991).

Detailed knowledge of how LHC proteins provide the unique environment, which enables pigments to efficiently collect and transfer energy, is still incomplete. LHC preparations which are mixtures of Cab gene products complicate biochemical and spectroscopic analyses (Green, 1988). In contrast, in vitro reconstitution with individual pigments and light-harvesting apoproteins synthesized in *Escherichia coli* (Cammarata & Schmidt, 1990; Paulsen et al., 1990) permits spectroscopic and biochemical evaluation of the pigment-binding characteristics of an individual LHC gene product. Moreover, in vitro mutagenesis of Cab genes allows delineation of the functions of specific amino acid residues. This approach therefore complements other work (Kohorn & Tobin, 1987; Kohorn, 1990) in which small amounts of mutagenized radiolabeled LHCP II proteins are either imported into chloroplasts or subjected to thylakoid insertion assays. Unequivocal conclusions regarding pigment-binding sites are not possible in the latter approach because the spectroscopic properties of the assembled products cannot be distinguished from those of the endogenous complexes.

We show by in vitro reconstitution that the product of a single LHC gene contains binding sites for Chls *a* and *b* and xanthophylls. The reconstituted complex is biochemically and spectroscopically similar to the mixture of LHC complexes isolated from thylakoids following LDS–PAGE. Toward identifying the pigment-binding domains of LHC complexes, deletion mutagenesis suggests an important structural role for His208 and delineates polypeptide regions which are (1) essential for pigment binding in vitro and (2) affect pigment-binding stability in general and Chl *a* binding in particular.

Materials and Methods

Preparation of Thylakoids and LHCP II Complexes. Broken chloroplasts were isolated from market spinach of 7-day-old pea seedlings by differential centrifugation as previously described (Tamura & Cheniae, 1985). TX100-derived LHCP II was prepared according to Arntzen et al. (1982) from spinach or pea chloroplasts treated with 0.8% TX100 at 0.8 mg of Chl/mL. Aliquots were frozen at −70 °C in gel sample buffer (100 mM Tris-HCl, pH 8.5/60 mM DTT/5 mM ACA/1 mM BAM). Alternatively, monomeric LHC was prepared by solubilizing thylakoid membranes with 2% LDS on ice (Delepelaire & Chua, 1981) and electrophoresing the mixture at 4 °C on nondenaturing "green gels" as described below. This preparation is referred to as native CP2. Delipidated polypeptides from thylakoids or TX100-derived LHCP II were obtained by extraction with 80% acetone and resolubilization in gel sample buffer (Plumley & Schmidt, 1987).

In Vivo Expression of an LHC Apoprotein in E. coli. AB96, a cDNA clone of a pea LHCP II gene encoding all but probably five amino acids at the NH_2 terminus of the M_r 26 000 mature apoprotein designated polypeptide 15 (Coruzzi et al., 1983), was a gift from Dr. A. Cashmore. The pDS12/RBSII-1 expression vector shown in Figure 1A was obtained from Dr. H. Bujard and differs from the pDS5 vector [described in Bujard et al. (1987)] by the inclusion of a prokaryotic ribosomal binding site at the *Eco*RI site in the pUC8-derived polylinker and stop signals for transcription and translation downstream of the *Hin*dIII site. Subcloning AB96 into the *Pst*I site of the pDS12/RBSII-1 expression plasmid allowed either in vivo or in vitro expression of the fusion protein derived from AB96, shown in Figure 1B. *E. coli* cells (DH5α, Bethesda Research Laboratories, or XL-1 Blue, Stratagene) were transformed by the method of Hanahan (1985). Recombinants were selected and characterized by (1) colony immunoscreening (Lyons & Nelson, 1984) using the immunoblotting protocol and antibodies described below; (2) colony hybridization (Sambrook et al., 1989) in which nitrocellulose membranes were probed with ^{32}P-labeled pAB96 generated by random primer labeling; and (3) sequencing (Sequenase, U.S. Biochemical Corp.) of plasmid DNA (Del Sal, 1989).

Amino- or carboxyl-terminal deletion mutants of AB96 were constructed from AB96/pDS-1 utilizing restriction enzyme digestion (*Avr*II, *Nco*I, *Ppu*MI) or by unidirectional exonuclease III digestion (Stratagene). Mutations were analyzed by restriction analyses and DNA sequencing. The derived amino acid sequences of selected mutants are presented in

Table I: Summary of Amino- and Carboxyl-Terminal Deletion Analyses of Various AB96 Constructs[a]

5' DELETION ANALYSES

AMINO ACIDS DELETED	5' VECTOR SEQUENCE	5' AB96 AND MUTANT SEQUENCES (10 20 30 40 50 60)	RECONSTITUTION
-	MRDPSTCS	TTKKVASSSSPWHGPDRVKYLGPFSGESPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIH	+
17-21	MRDPSTCS	TTKKVASSSSPWHGPD-----GPFSGESPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIH	+
1-10	MRD	PWHGPDRVKYLGPFSGESPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIH	+
1-20	MRIRR	LGPFSGESPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIH	+
1-49	MR	DPETFAKNRELEVIH	+
1-57	MRDPSTCS	RELEVIH	-

3' DELETION ANALYSES

AMINO ACIDS DELETED		5' VECTOR SEQUENCE	5' AB96 AND MUTANT SEQUENCES	3' AB96 AND MUTANT SEQUENCES (200 210 220 228)	3' VECTOR SEQUENCE	RECONSTITUTION
-	-	MRDPSTCS	TTKKVASSSSPWHGPDRVKYL-----	IVTGKGPLENLADHLADPVNNNAWSYATNFVPGK*	-	+
1-10	219-228	MRD	PWHGPDRVKYL-----	IVTGKGPLENLADHLADPVNNNAW	AKLN*LSLDSC*	+
1-10	214-228	MRD	PWHGPDRVKYL-----	IVTGKGPLENLADHLADPV	SQA*	+
1-10	213-228	MRD	PWHGPDRVKYL-----	IVTGKGPLENLADHLADP	AKLN*LSLDSC*	+
1-10	204-228	MRD	PWHGPDRVKYL-----	IVTGKGPLE	PSLIS*	-
1-10	201-228	MRDPSTCS	TTKKVASSSSPWHGPDRVKYL-----	IVTGKG	QLI*	-

*: Non-Sense Codon

[a] Amino acid sequences derived from DNA sequence analyses are shown for AB96 and derivative deletion mutant constructs and flanking vector encoded regions. Amino acids deleted from AB96 and the ability to reconstitute are indicated. See Materials and Methods for details. Structural assignments for AB96 were derived from Pichersky and Green (1990), Evans and Nugent (1991), and Butler and Kühlbrandt (1991) and are indicated below the wild-type sequences. The heavy bars correspond to the hydrophobic core of the transmembrane α-helix. Light bars indicate the charged α-helical extensions. Regions of β-turn are denoted by dashed lines beneath the AB96 sequence.

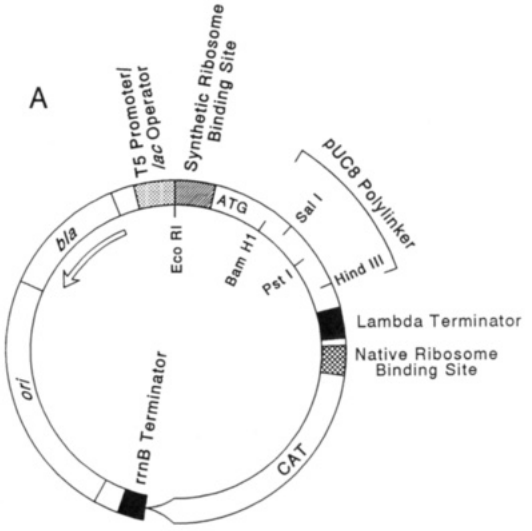

FIGURE 1: Expression vector construction (A) and deduced N-terminal amino acid sequence (B) of the AB96 fusion protein derived from in vivo expression in pDS12/RBSII-1 by *E. coli*. See Materials and Methods for description of the pDS12/RBSII-1 derivative of the pDS5 vector (Bujard et al., 1987).

Table I. Numbering of the AB96 amino acid sequence is such that the first threonine residue encoded by the nucleotide sequence published by Coruzzi et al. (1983) corresponds to residue number one.

E. coli transformants harboring the pDS12/RBSII-1 expression vector, either with or without various AB96 constructs, were grown to early log phase on LB media supplemented with 50 μg/mL ampicillin and 12.5 μg/mL tetracycline. Expression was induced by 1 mM IPTG with subsequent growth for 20 min to 6 h at 37 °C. Cells were chilled and harvested by centrifugation for 10 min at 12000*g* and washed once in wash buffer (100 mM Tris-HCl, pH 8.5/5 mM EDTA/50 mM NaCl/5 mM ACA/1 mM BAM). Equivalent numbers of cells were lysed by resuspension in 100 mM Tris-HCl, pH 8.5/25 mM DTT/12% sucrose/2% LDS/5 mM ACA/1 mM BAM. After 10 min of incubation at 0 °C, the viscous cell lysates were sonicated (5–7 pulses; 20% cycle; output = 10) (Heat Systems/Ultrasonic) and heated (100 °C, 1 min). Insoluble material was pelleted by centrifugation for 5 min at 12000*g*. The whole-cell lysates of *E. coli* (containing AB96 apoproteins approximately equivalent to the amount of total LHC proteins present in thylakoids corresponding to 50 μg of Chl) were then subjected to the reconstitution procedure described below or frozen at −70 °C. Alternatively, the fusion proteins were partially purified as described by Nagai and Thogersen (1987) and frozen at −20 °C in gel sample buffer. Approximately 50 μg of purified protein was used in a typical 50-μL reconstitution assay.

Pigments. Total pigment preparations and individual xanthophylls from thylakoids or TX100-derived LHC were obtained from 80% (v/v) acetone (buffered with $MgCO_3$) extracts that were partitioned into ether (Plumley & Schmidt, 1987). Purified Chl *a* and *b* (Sigma Chemical Co.) were

dissolved in diethyl ether and stored at −20 °C under argon. Since the pigment compositions of thylakoids from both pea and spinach are essentially identical, pigments and proteins from either plant can be used interchangeably for LHC reconstitution.

LHCP II Reconstitution from Thylakoids and LHC Apoproteins. LHCP II reconstitution from thylakoids or LHC apoproteins by the freeze/thaw method and subsequent nondenaturing LDS–PAGE at 4 °C were essentially as described by Plumley and Schmidt (1987). Thylakoids or LHC apoproteins equivalent to approximately 50 μg of Chl were reconstituted with the total pigment preparations (50 μg of Chl) that were adjusted to a Chl *a*/*b* ratio of 1.3 with supplemental Chl *b*. Alternatively, individually purified pigments were used in the ratios described by Plumley and Schmidt (1987). Aliquots of pigments (50–60 μg of Chl) in ether/ethanol were added directly to the thylakoids/apoproteins solubilized in gel sample buffer plus 2% LDS/12.5% (w/v) sucrose. Reconstitution controls consisted of reconstituted samples heated (100 °C, 1 min) just prior to electrophoresis or solubilized membrane/apoprotein samples lacking pigments but otherwise similarly exposed to ether/ethanol and three cycles of freeze/thaw. Unstained electrophoretic profiles of the green gels were photographed directly to visualize the reconstituted CP2 pigment protein complexes.

Analyses of Reconstituted CP2 Recovered from LDS–PAGE. After LDS–PAGE at 4 °C (Schmidt & Mishkind, 1983), reconstituted CP2 pigment–protein complexes were recovered by excision of the pigmented bands and homogenization of the polyacrylamide in 10 mM Tris-HCl, pH 8.0/5 mM ACA/1 mM BAM, slow mixing for 30 min (4 °C), and pelleting the polyacrylamide by 25 min of centrifugation at 50000*g* and filtration through a 0.45 μM porosity membrane. All steps were performed at 4 °C with minimal exposure to light. Recovered CP2 samples were analyzed immediately or frozen at −70 °C. When necessary, sample concentration was achieved in a stirred ultrafiltration unit (Amicon) using a PM10 membrane.

Absorption, fluorescence, and CD spectra of the recovered CP2 complexes (less than or equal to 10 μg of Chl/mL) were measured at room temperature. Absorption spectra were recorded with a Shimadzu UV265 spectrophotometer using a 0.2-nm slit width. Fluorescence spectra corrected for photomultiplier response were determined with an SLM Aminco SPF-500 fluorometer using 4-nm emission and excitation bandwidths. CD spectra were recorded on a Jasco J-500C spectropolarimeter equipped with a Jasco DP-501N data processor using a 2-nm slit width. Presented CD spectra represent the average of four to eight scans.

The pigment composition of recovered CP2 was monitored by TLC separation of the pigments extracted with 80% (v/v) acetone buffered with $MgCO_3$. Pigments were partitioned into ether, dried under vacuum, and redissolved in a minimal volume of ether. This extract was spotted onto Whatman LKC-18 reversed-phase TLC plates which were then developed in 114/56/30 methanol/ethyl acetate/water. Alternatively, the pigment extracts were analyzed by HPLC on an Altex Ultrasphere-ODS column using a linear gradient of 75–100% acetone generated over 8 min at a flow rate of 1 mL/min. Separation profiles were monitored by absorbance at 436 nm and quantitated by integration of the peak areas. Comparisons of pigment levels were made on the basis of absorption ratios relative to the amount of Chl *b*. Polypeptide profiles from LDS–PAGE were either visualized by staining with Coomassie Blue R-250 or electrophoretically transferred to nitrocellulose using the Transblot (Bio-Rad; tank buffer, 25 mM Tris/192 mM glycine/20% v/v methanol/0.02% w/v SDS) transfer system according to the manufacturer's instructions. Transfer efficiency was monitored by staining the nitrocellulose membrane with 0.2% Ponceau Red 3R (Nakamura et al., 1985).

Immunoblots were visualized using alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma Chemical Co.) as previously described (Blake et al., 1984; Greer et al., 1986) except that the blocking and washing buffers contained fish skin gelatin in place of bovine serum albumin. The primary antibody to LHC was a polyclonal antibody raised against *Chlamydomonas reinhardtii* thylakoid polypeptide 11 [prepared as described in Plumley and Schmidt (1983)] which recognizes all major LHCP II polypeptides of *Chlamydomonas reinhardtii*, spinach, and pea.

Results

Reconstitution of a Single LHC Gene Product Expressed in E. coli. LHCP II can be reconstituted in vitro from thylakoid apoproteins to yield pigment–protein complexes with spectroscopic properties nearly identical to those of "native" CP2 complexes isolated from LDS–PAGE (Plumley & Schmidt, 1987). The reconstitution process exhibits an absolute requirement for all of the major individual components (LHCP II apoproteins, Chls *a* and *b*, lutein, violaxanthin, and neoxanthin) of native LHCP II. However, LHCP II preparations contain a mixture of products of the Cab multigene family. Knowledge of the roles of the individual Cab gene products with regard to pigment binding, including elucidation of the precise mechanisms of pigment ligation, is required for a thorough understanding of the physiological dynamics of LHCP II. Progess toward reconstituting individual apoproteins has been achieved using PAGE-purified single LHC apoproteins (Plumley & Schmidt, 1987) or immunoprecipitates obtained with monospecific monoclonal antibodies (Cammarata & Schmidt, 1990). Another approach is to characterize the pigment-binding properties of individual LHCP II apoproteins which are expressed in *E. coli* from cloned genes (Cammarata & Schmidt, 1990; Paulsen et al., 1990).

AB96, a cDNA clone encoding most of a mature type I LHCP II apoprotein from pea (Corruzzi et al., 1983), was subcloned into the pDS12/RBSII-1 vector (Bujard et al., 1987) to permit both in vivo and in vitro expression of the fusion protein as illustrated in Figure 1B. Eight hydrophilic vector-encoded amino acids are appended to the amino terminus of the apoprotein encoded by AB96. Either whole cell lysates of *E. coli* or proteins from partially purified inclusion bodies were utilized for reconstitution studies.

The nondenaturing green gel shown in Figure 2A demonstrates that a single LHCP II gene product contains sufficient information to be reconstituted with pigments. Lanes 2 and 4 contain LDS–PAGE-stable pigment–protein complexes (CP2) reconstituted from AB96 expressed in *E. coli* and total thylakoid apoproteins, respectively. The eight amino acid NH_2-terminal extension present in the fusion protein is not detrimental to reconstitution of pigment binding. Furthermore, no pigment–protein complexes are observed for control samples lacking LHCP II apoproteins (lysates of *E. coli* harboring pDS vector either without AB96, lane 6, or with AB96 in an inverse orientation, lane 1) or from a reconstituted AB96-containing lysate or thylakoids boiled just prior to electrophoresis (lanes 3 and 5). Therefore, nonspecific pigment-binding artifacts do not occur as is also confirmed by spectroscopic analyses (see below).

Pigment requirements for reconstitution of the AB96 apoprotein are similar to those observed for reconstituting total

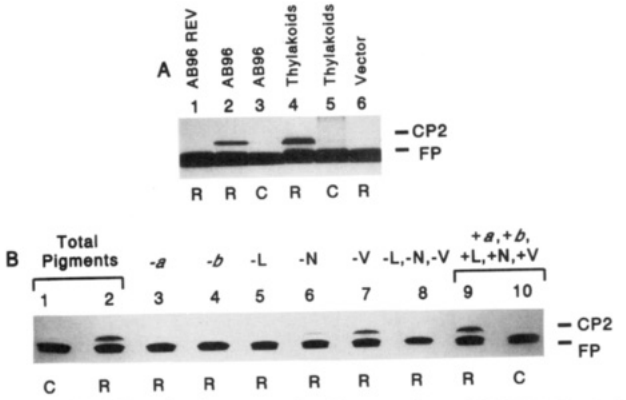

FIGURE 2: Unstained green gels of reconstituted LHCP II. (A) Reconstitution of the AB96 gene product. Whole-cell lysates of *E. coli* expressing the AB96 gene product and delipidated spinach thylakoid membrane proteins were reconstituted (R) as described in Materials and Methods with total spinach pigments supplemented with Chl *b* (Chl *a*/*b* = 1.3). Each lane contains 35 μL of a 50-μL reconstitution mixture comprised of 50 μg of AB96 apoproteins plus total spinach pigments corresponding to 47 μg of Chl and 17 μg of Chl *b* (Chl *a*/*b* = 1.3). Control samples (C) were boiled for 1 min prior to electrophoresis. Additional controls for pigment-binding specificity consisted of lysates of cells with the expression vector lacking the AB96 gene (lane 6) or with the AB96 gene in reverse orientation in the expression vector (lane 1). FP, free pigment at the electrophoretic front; CP2, LHCP II pigment–protein complex. (B) Reconstitution of the AB96 gene product with mixtures of individual pigments. Partially purified AB96 apoproteins synthesized in *E. coli* were reconstituted with total spinach pigments supplemented with Chl *b* as described above (lanes 1 and 2) or with a mixture of individually purified spinach pigments (lanes 9 and 10) as described by Plumley and Schmidt (1987). Various components of the pigment mixture were omitted from the samples shown in lanes 3–8. a, Chl *a*; b, Chl *b*; L, lutein; V, violaxanthin; N, neoxanthin. Other abbreviations are as above.

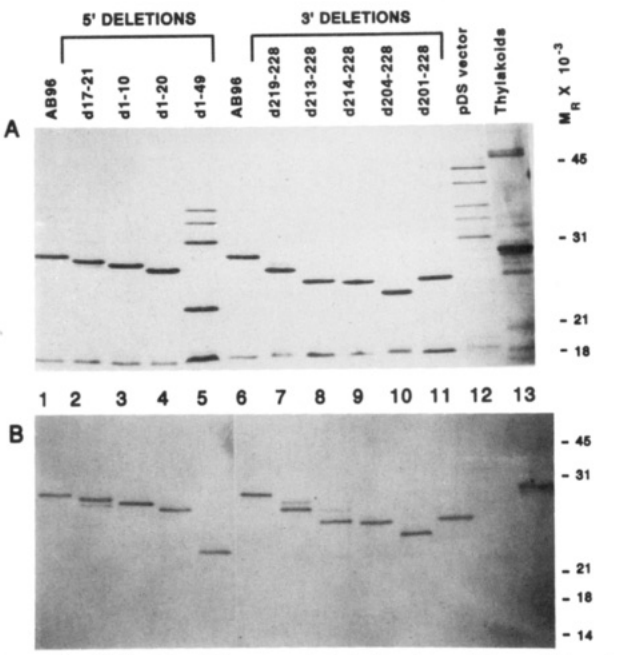

FIGURE 3: Stained and immunodetected polypeptide profiles of partially purified AB96 deletion mutant gene products. (A) Coomassie-stained polypeptide profile. Partially purified AB96 deletion mutant gene products expressed in *E. coli* were solubilized with LDS, electrophoresed on a 10–20% polyacrylamide gel, and visualized by staining with Coomassie Brilliant Blue R. Numbers indicate amino acids deleted from various AB96 constructs. Relative molecular mass is indicated. (B) Immunoblot. Electrophoretically fractionated polypeptides were transferred to nitrocellulose and immunodetected as described in Materials and Methods. Other details are as described above.

thylakoid LHCP II gene products (Plumley & Schmidt, 1987). Lane 9 of Figure 2B shows that the AB96 gene product can be reconstituted with purified Chls *a* and *b* plus the xanthophylls lutein, violaxanthin, and neoxanthin. As concluded previously, a CP2 pigment–protein complex can be formed from the AB96 apoprotein in the absence of acyllipids. Omission of either Chl species or of lutein prevents recovery of reconstituted CP2 (lanes 3, 4, 5, and 8). Although it was previously concluded that neoxanthin was essential for reconstitution (Plumley & Schmidt, 1987), data in lane 6 of Figure 2B suggest that extremely small amounts of CP2 can be reconstituted without this xanthophyll. Likewise, reconstitution can proceed suboptimally in the absence of violaxanthin (lane 7) provided that lutein amounts are correspondingly increased (Plumley & Schmidt, 1987).

Since all of the major pigment-binding sites among the thylakoid population of LHCP II are also present on the AB96 protein, the effects of amino- and carboxyl-terminal deletions on the pigment reconstitution process can be examined. Deletions blocking reconstitution altogether probably affect regions that are structurally important for pigment binding and/or protein conformation. In contrast, mutations which result in decreased yields of reconstituted products could arise from incomplete or perturbed pigment assembly, in which cases the changes can be assessed spectroscopically.

Reconstitution of Gene Products from AB96 Deletion Mutants. Deletions of the 3′ and 5′ regions of the AB96 coding sequence were constructed for bacterial expression. Table I shows the amino acid sequences encoded by AB96 and its deletion mutants including translatable, vector-encoded 3′ and 5′ flanking regions. According to structural models (Karlin-Neumann et al., 1985; Bürgi et al., 1987; Pichersky & Green, 1990; Kühlbrandt & Wang, 1991), the 5′ deletions affect NH_2-terminal surface-exposed regions approaching the stroma–membrane interface and the first of three membrane-spanning α-helices. The two largest 5′ deletions eliminate a conserved "hook" region (Kühlbrandt & Wang, 1991) which may arise from a β-turn structure (Pichersky & Green, 1990). The 3′ deletions affect the COOH-terminal surface-exposed regions at the lumen–membrane interface and the third membrane-spanning α-helix. Part of the latter region may comprise a short amphipathic α-helix which is conserved among most classes of plant light-harvesting complexes (Pichersky & Green, 1990; Green et al., 1991). Most of the 3′ deletion constructs also contain a short 5′ deletion (10 amino acids) which facilitated construction of the mutant genes. Comparisons with the parent 5′ deletion construct show that this additional modification has no effect on pigment reconstitution (see below).

The apoproteins of AB96 and its mutant derivatives were efficiently synthesized and accumulated in *E. coli*, as can be seen from the Coomassie-stained LDS–PAGE profile and the immunoblot shown in parts A and B, respectively, of Figure 3. There is similar accumulation of mutant apoproteins which lack 57 amino-terminal residues (data not shown). In all but one case, the observed relative mobilities correlate with the predicted sizes of the fusion proteins. However, protein from the 3′ deletion mutant 201–228 migrates more slowly during LDS–PAGE than would be predicted from its size as deduced by DNA sequence analysis. This may reflect a loss of hydrophilic amino acids, exposure of a hydrophobic region, and/or an abnormal conformation during nondenaturing electrophoresis. In some cases, relatively small amounts of higher (lanes 7 and 8 of Figure 3B) or lower (lane 2 of Figure 3B) molecular weight LHC apoproteins are also synthesized.

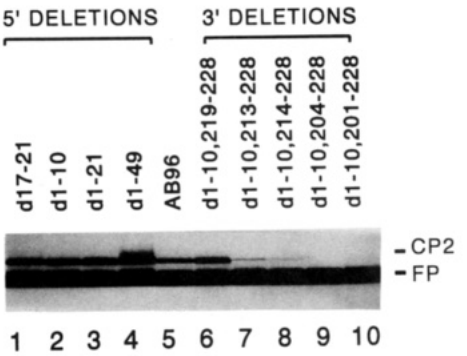

FIGURE 4: Reconstitution of the AB96 deletion mutant gene products. Unstained gel as described for Figure 2. Numbers indicate amino acids deleted from the various constructs. Reconstitution controls for this experiment are shown in Figure 2A.

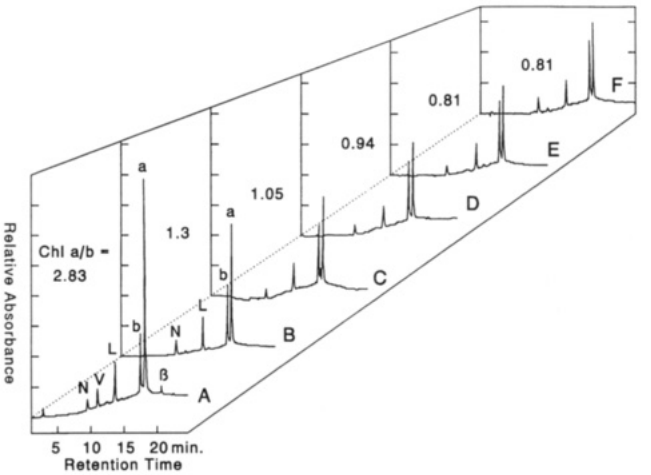

FIGURE 5: HPLC analysis of pigments reconstituted into the AB96 deletion mutant gene products. Pigments from recovered CP2 complexes were extracted in 80% acetone and analyzed by HPLC as described in Materials and Methods. The traces are presented on the basis of equivalent amounts of Chl *b*. a, Chl *a*; b, Chl *b*; L, lutein; V, violaxanthin; N, neoxanthin; β,β-carotene. (A) spinach thylakoids; (B) native CP2 from pea thylakoids; (C) reconstituted spinach total thylakoid apoproteins; (D-F) reconstituted gene products of AB96 and the NH_2- and COOH-terminal deletion mutants lacking amino acids 1–49 and 1–10/213–228, respectively.

The former cases correlate with the occurrence of a particular 3′ flanking vector sequence in which the first nonsense codon encountered during translation (TAG) might be occasionally disregarded by the *E. coli* translation apparatus. The carboxyl-terminal extension is indicated in Table I. For the smaller product observed for the 5′ deletion mutant in lane 2, partial proteolysis seems most likely. In all cases, however, the abundance of secondary LHC gene products is quite low: they are clearly visualized only by highly sensitive techniques such as immunoblotting. The Coomassie-stained profiles indicate that the secondary LHC apoproteins represent less than 10% of the accumulated LHC gene product. The additional Coomassie-stained bands in lane 5 of Figure 3A are contaminants of *E. coli* origin (see Figure 3B).

Figure 4 shows an unstained green gel in which the various deletion mutant proteins were subjected to reconstitution with pigments. The presence of CP2-like pigment–protein complexes in lanes 1–4 indicates that deletion of up to 49 amino acids from the amino terminus of the AB96 sequence does not affect the ability of the mutant proteins to bind pigments. Figure 4 does not show the reconstitution test of the products of a deletion mutant lacking 57 NH_2-terminal amino acids because no reconstituted CP2 could be obtained from these proteins. It appears that the NH_2-terminal 8 amino acids of the first membrane-spanning α-helix (Kühlbrandt & Wang, 1991) are necessary for reconstitution. It should also be noted that mutants with large deletions frequently reconstitute with lower efficiency and are candidates for complexes with altered pigment-binding characteristics.

The 3′ deletion analyses suggest that a 9 amino acid region (204–212) near the COOH terminus is essential for reconstitution or stability of the CP2 complex. Elimination of 25 or more (204–228; 201–228) of the COOH-terminal residues blocks recovery of reconstituted CP2 entirely (lanes 9 and 10), whereas deletion of 16 (213–228) or fewer amino acids is permissive for pigment binding (lanes 6–8). However, the mutant proteins used in lanes 7 and 8 (COOH-terminal truncations of 15 and 16 amino acids, respectively) always exhibit a greatly lowered efficiency of reconstitution. Nonetheless, the low levels of reconstitution in these cases are still sufficient for subsequent analyses (see below). This indicates that the Asn and/or Trp residues between positions 214 and 218 either comprise a discrete pigment-binding site or play an important stabilizing role for the complex. The 9 amino acid region (residues 204–212) contains possible pigment ligands (His and Asn residues) as well as potentially important structural determinants (Asp and Pro residues). Deletion of this region also eliminates the putative amphipathic α-helix (Pichersky & Green, 1990). Similar results for other mutant constructs differing only in the COOH-terminal vector-encoded sequence (data not shown) negate the possibility that the observed effects on reconstitution are due to the introduction of extraneous amino acid sequences at the COOH terminus.

Pigments of Reconstituted Complexes. Analyses of the composition and structural organization of pigments in the CP2-like complexes were facilitated by preparative reconstitution and recovery procedures. Profiles of pigments associated with LHCP II complexes can be resolved by HPLC and are shown in Figure 5. Trace A illustrates the levels of Chls *a* and *b*, β-carotene, and the major xanthophyll components lutein, violaxanthin, and neoxanthin present in spinach or pea thylakoids. All pigments except β-carotene are necessary for reconstitution of LHCP II (Plumley & Schmidt, 1987) and, with the additional exception of violaxanthin, are present in native CP2 isolated by LDS–PAGE (4 °C) of nonheated solubilized thylakoids (trace B). The near absence of violaxanthin in CP2 is in contrast to its abundance in thylakoids or TX100-derived LHCP II [data not shown; see also Braumann et al. (1982)]. Since the yields of this pigment are diminished by prolonged or denaturing electrophoretic conditions (our unpublished observations; Braumann et al., 1982), violaxanthin cannot be quantitated in CP2 preparations. Spectrophotometric determination of Chl *a*/*b* ratios of pigments solubilized in 80% acetone from native and reconstituted CP2 reveals a range of values from 0.7 to 1.3, but the ratio is typically near 1. Clearly, dissociation/degradation during electrophoresis are important determinants for the final pigment composition of CP2 preparations.

The comparison of the reconstituted CP2 complexes in Figure 5 shows that, with the exception of violaxanthin, all of the pigments present in native CP2 (trace B) are also present in complexes reconstituted from delipidated light-harvesting apoproteins (trace C), the gene products from AB96 (trace D), and the apoproteins from AB96 deletion mutants (traces E and F). The relative stoichiometries between Chls *a* and *b* and xanthophylls are similar for native versus reconstituted AB96 complexes. Data are shown only for the largest of the NH_2- and COOH-terminal deletions which are still recon-

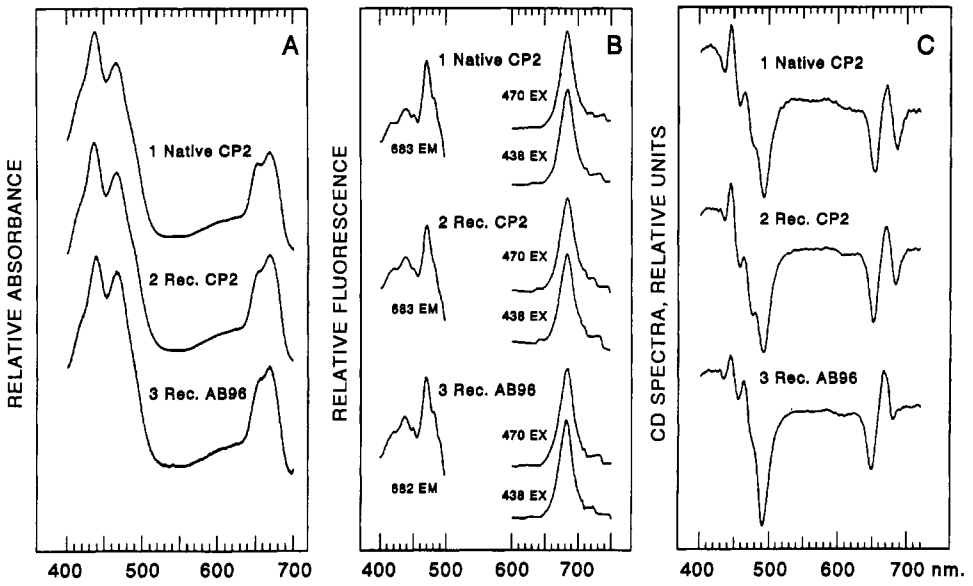

FIGURE 6: Spectroscopic characterization of reconstituted AB96 gene product in comparison to native CP2. Recovered CP2 complexes were measured at approximately 10 μg of Chl/mL as described in Materials and Methods. (1) Native CP2 from nonheated LDS-solubilized pea thylakoids; (2) CP2 reconstituted from total thylakoid apoproteins; (3) CP2 reconstituted from AB96 expressed in *E. coli*. (A) Visible absorption spectra. Spectra were normalized to the absorbance maximum at 438 nm. (B) Fluorescence spectra. Excitation and emission wavelengths are indicated. Excitation spectra were recorded at the wavelength of fluorescence maximum. Spectra were normalized to the fluorescence maximum. (C) Circular dichroism. Spectra are the average of eight scans. Spectra were normalized to the negative signal at 490 nm attributed to Chl *b*.

stitutable, but these and the products of all intermediate deletion constructs have pigment compositions qualitatively similar to that of AB96. Only the Chl *a*/*b* ratios are altered for the truncated AB96 polypeptides. Reconstituted constructs lacking either 49 NH_2-terminal amino acids or 16 COOH-terminal amino acids have, in the best cases, Chl *a*/*b* ratios diminished by 10–20% (to approximately 0.8–0.9). Constructs with lesser deletions typically show no significant difference from AB96 (0–10% reduction). However, when recovered after prolonged electrophoresis, the Chl *a*/*b* ratio of even AB96 can be as low as 0.7. This decrease most likely reflects dissociation of Chl *a* because xanthophyll/Chl *b* ratios remain relatively constant. Thus, the data in Figure 5 indicate that portions of the NH_2 and COOH termini of AB96 (amino acids near 49 and 213) may be important for stable binding of perhaps 1 or 2 Chl *a* molecules in the CP2 complex. Moreover, the analyses suggest that these portions of the AB96 polypeptide affect the stability of a labile fraction of Chl *a* in the complex. Spectroscopic data reinforce this supposition.

Spectroscopic Analyses of CP2 Reconstituted from AB96 Gene Products. The spectroscopic analyses of Figure 6 compare pigment organization in reconstituted AB96 apoproteins, native CP2, and CP2 reconstituted from total LHC apoproteins. Qualitatively similar absorption spectra are observed (Figure 6A) with complexes eluted from green gels. Small variations in the relative amounts of Chl *a* (A_{max} at 438 and 672 nm) and Chl *b* (A_{max} at 471 and 652 nm) are observed for native (Green, 1988) and reconstituted LHCP II (Plumley & Schmidt, 1987). Fluorescence spectroscopy of the reconstituted AB96 apoprotein demonstrates intramolecular energy transfer properties which are qualitatively similar to those of native CP2 since the same 682-nm fluorescence emission maximum is observed when Chl *a* or Chl *b*, respectively, are excited at 438 or 470 nm (Figure 6B, right). Excitation spectra for native and reconstituted CP2 complexes (Figure 6B, left) confirm that both Chl *a* and Chl *b* can contribute to the 682-nm Chl *a* fluorescence.

The organization of pigments reconstituted into LHC apoproteins also can be assessed by circular dichroism (CD) spectroscopy. A CD signature characteristic of native CP2, derived from LDS-solubilized nonheated thylakoids, is shown in Figure 6C (trace 1) and is the standard for the pigment interactions and orientations which occur in CP2. CD spectra of CP2 reconstituted from total LHC apoproteins (trace 2) are nearly identical and are comparable to those published for similar preparations (Van Metter, 1977; Ide et al., 1987; Plumley & Schmidt, 1987). The transition from the positive signal at 670 nm to the prominent negative signal at 650 nm is believed to be characteristic of a specific organization responsible for exciton splitting between a trimer of Chl *b* molecules (Van Metter, 1977; Shepanski & Knox, 1981). The negative signal near 683 nm may represent specialized Chl *a* in LHCP II (Van Metter, 1977). The complex CD spectrum in the Soret region also indicates similar organization of the contributing Chl *a* and Chl *b* absorption moieties in native CP2 and reconstituted total LHC apoproteins. Trace 3 of Figure 6C demonstrates that the reconstituted AB96 gene product has qualitatively identical pigment–protein and pigment–pigment interactions, insofar as they are detected by CD spectroscopy, as those of native CP2. Thus, the AB96 LHC gene product contains sufficient information to establish the pigment orientations necessary for the complex Soret characteristics and exciton splitting between Chl *b* molecules. The CD spectrum of CP2 reconstituted from the AB96 apoprotein differs quantitatively by having slightly diminished signal strengths at 475, 650, and, most notably, 683 nm (relative to the most prominent Chl *b* signal at 490 nm). The decreased signal strength at 683 nm in reconstituted AB96 CP2 correlated with the small but consistent decrease in the Chl *a*/*b* ratio as compared to that of native CP2. Clearly, in vitro reconstitution of a single species of LHC apoprotein synthesized by *E. coli* preserves the integrity of the major pigment–protein and pigment–pigment interactions found in native CP2.

Spectroscopic analyses of the reconstituted AB96 mutant gene products are depicted in Figures 7 and 8. No anomalies in the visible absorption spectra of reconstituted complexes are evident for any of the mutated gene products as compared

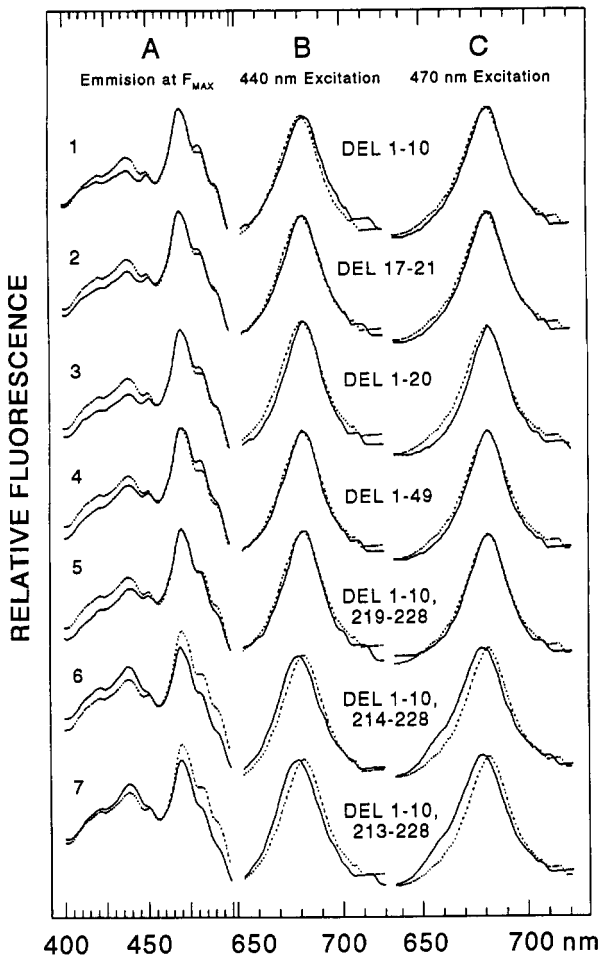

FIGURE 7: Fluorescence spectroscopy of the reconstituted gene products of the NH_2- and COOH-terminal deletion mutants of AB96. See Materials and Methods and Figure 6 for details. (A) Excitation spectra recorded at the wavelength of the fluorescence maximum; (B) emission spectra from 438-nm excitation (Chl *a*); (C) emission spectra from 470-nm excitation (Chl *b*). Solid lines: NH_2-terminal deletions 1, 1–10; 2, 17–21; 3, 1–20; and 4, 1–49 and COOH-terminal deletions 5, 1–10/219–228; 6, 1–10/214–228; and 7, 1–10/213–228. Dotted lines: AB96 control spectra.

to that of AB96 (data not shown). However, comparison of the fluorescence spectra of Figure 7 reveals alterations of the pigment organization reconstituted from the AB96 deletion mutant gene products lacking either 15 or 16 COOH-terminal amino acids. All other AB96 constructs have fluorescence characteristics nearly identical to those of AB96 (dotted lines). The loss of 15 or 16 amino acids from the COOH terminus disrupts the normal pigment–pigment interactions as evidenced by a 3–4-nm blue shift of the fluorescence maximum and the appearance of a fluorescence shoulder at approximately 660 nm when Chl *b* is excited at 470 nm (Figure 7, traces 6C and 7C). The latter observation indicates that a fraction of the Chl *b* is disconnected with regard to energy transfer to Chl *a*. The excitation spectra (traces 6A and 7A) support this conclusion because there is relatively more contribution to the observed 678-nm fluorescence from Chl *a* and less from Chl *b* upon comparison to the AB96 control (dotted line). A change in Chl *a* organization is also evident from a blue shift of the fluorescence maximum when 438-nm light is used for excitation (traces 6B and 7B). Thus, amino acids 214–218 at the carboxyl terminus appear to influence pigment–pigment interactions within the CP2 complex.

In Figure 8, CD spectra of reconstituted mutant gene products are compared to that of AB96 (dotted lines). With one exception, all of the reconstitutable mutant gene products

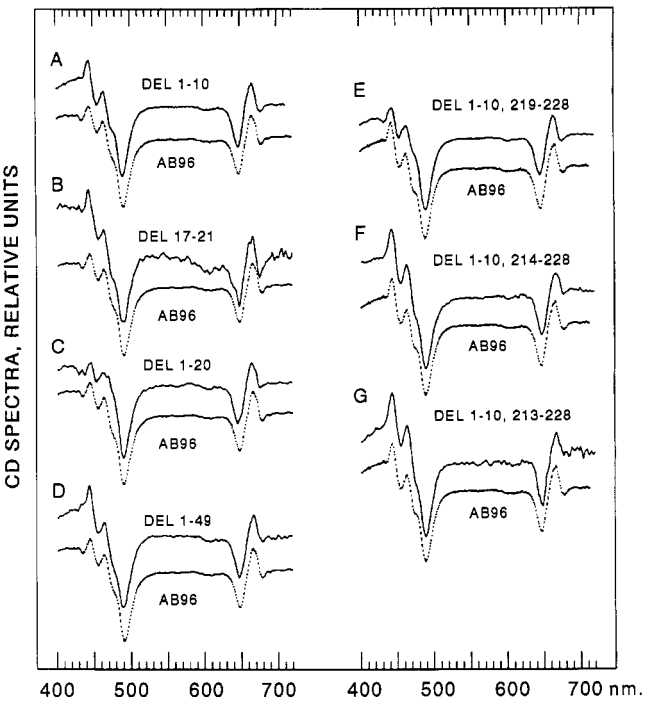

FIGURE 8: Circular dichroism of the reconstituted gene products of the NH_2- and COOH-terminal deletion mutants of AB96. Spectra represent the average of eight scans and are normalized to the negative signal at 490 nm attributed to Chl *b*. Solid lines: NH_2-terminal deletions A, 1–10; B, 17–21; C, 1–20; and D, 1–49 and COOH-terminal deletions E, 1–10/219–228; F, 1–10/214–228; and G, 1–10/213–228. Dotted lines: AB96 control traces.

yield complexes with CD spectra qualitatively similar to that of AB96. The construct lacking 16 COOH-terminal amino acids (trace G) does not possess a negative 683-nm CD signal, suggesting that a specific form of Chl *a* is not present. Again, either the stability or direct binding of a uniquely organized species of Chl *a* is affected by amino acids 214–218 at the COOH terminus. In the absence of this pigment, some of the bound Chl *b* appears to become disconnected from the energy transfer pathway. An alternative hypothesis is that amino acids 214–218 affect Chl *b* organization directly and that the overall perturbation of the CP2-like complex results in loss of the Chl *a* site which is defined by the 683-nm CD signal.

Discussion

Reconstitution of a Single LHC Apoprotein. Spectroscopic techniques have been used to explore the pigment organization of LHCP II (Van Metter, 1977; Shepanski & Knox, 1981; Ide et al., 1987; Lin & Knox, 1988). The Van Metter–Shepanski–Knox model describes the energy levels and transfer pathways from three strongly coupled Chl *b* molecules with C_3 symmetry to three weakly coupled Chl *a* molecules. Further progress is being made in elucidating the macromolecular structure of LHCP II at the three-dimensional level through electron crystallography, including the suggestion that the Mg atom of chlorophyll may be distant from amino acid side chains (Kühlbrandt & Wang, 1991). However, LHCP II preparations contain multiple Cab gene products, differing in amino acid sequence, whose individual contributions to the structure and function of LHCP II are unknown (Chitnis & Thornber, 1988; Green, 1988; Green et al., 1991). In vitro import (Schmidt & Mishkind, 1986; Keegstra et al., 1989) and thylakoid insertion (Cline, 1986; Chitnis et al., 1987; Yalovsky et al., 1990) methodologies used in conjunction with mutagenesis of LHCP II proteins reveal insight into the maturation and assembly steps (Kohorn & Tobin, 1987; Kohorn,

1990), but these approaches are not amenable to spectroscopic characterization of pigment-binding sites. Thus, little is known of the specific modes of pigment binding within the protein framework or how each protein species might ligate specific pigments in relation to unique antenna functions (Chitnis & Thornber, 1988; Green, 1988).

We have addressed these problems by reconstituting a single species of LHC apoprotein (Cammarata & Schmidt, 1990) and examining the characteristics of deletion mutant gene products within the context of structural models (Karlin-Neumann et al., 1985; Bürgi et al., 1987; Pichersky & Green, 1990; Kühlbrandt & Wang, 1991). It is demonstrated that a single species of LHC apoprotein has binding sites for Chls *a* and *b*, plus the xanthophylls lutein, neoxanthin, and probably violaxanthin. Omission of one or more of these components causes instability or impairs reassembly of CP2. The compositions (Chl *a*, Chl *b*, lutein, neoxanthin) and relative stoichiometries of pigments in native and reconstituted CP2 complexes are similar. In addition, the relative ratio of LHC apoprotein to Chl *b* is similar for native CP2 and the reconstituted AB96 apoprotein (data not shown). Excitation coupling of Chl *b* with Chl *a* and exciton splitting between Chl *b* molecules are confirmed by spectroscopic analyses. We conclude that, within the limitations imposed by LDS–PAGE, in vitro reconstitution of the AB96 product restores the qualities of the pigment–pigment and pigment–protein interactions found with LHCP II formed in vivo. Similar results were also observed by Paulsen et al. (1990) using the pea AB80 gene encoding an LHC precursor protein, and a deletion mutant thereof which lacked 12 amino acids from the mature protein. Interestingly, the complex formed with the AB96 apoprotein exhibits a Chl *a*/*b* ratio that differs somewhat from that of TX100-derived LHC and native CP2. Hence, the AB96 complex may have specialized light-harvesting properties in vivo.

Effects of Deletion Mutagenesis on Pigment Binding and Organization in the AB96 Gene Product. With the availability of a reconstitution system for individual LHCP II gene products, molecular manipulation of individual genes can be used to assess pigment-binding sites and correlate polypeptide domains with function. Models based on hydropathicity and secondary structure predictions (Chitnis & Thornber, 1988; Pichersky & Green, 1990; Green et al., 1991) suggest that, for AB96, the NH_2- and COOH-terminal α-helix border regions at the membrane–surface interfaces are, respectively, near amino acids 60 and 202. Electron crystallographic studies of pea LHCP II confirm the predictions of 3 membrane-spanning helices and further indicate that the first and third (B and A) are unusually long and extend beyond the membrane (Evans & Nugent, 1991; Kühlbrandt & Wang, 1991; see Table I). Interestingly, a portion of the extended helices are not as highly conserved among the different classes of Cab genes (Pichersky & Green, 1990). Just beyond these regions, conserved β-turn motifs correlate with two stromal exposed "hooks" that exhibit 2-fold symmetry and comprise amino acids 29–52 and 152–166 in AB96. The lumenally-exposed kink in the lower portion of helix A corresponds to a putative amphipathic helix (Pichersky & Green, 1990) which would be at amino acids 203–212 in the AB96 apoprotein.

Whereas deletion of 10 amino acids from the COOH terminus or 20 amino acids from the NH_2 terminus of AB96 (unconserved regions predicted to be surface-exposed) have no discernible effects on reconstitution, extending NH_2- and COOH-terminal deletions into transmembrane α-helices B and A by removal of 57 and 25 amino acids, respectively, blocks recovery of reconstituted CP2 complexes altogether. Since a deletion mutant product lacking only 49 NH_2-terminal amino acids can be reconstituted, an 8 amino acid segment of helix B (50–57) is critical for pigment binding. The COOH-terminal mutation defines the putative amphipathic helix as a structure critical for stable pigment binding. Whereas the unconserved His13 at the NH_2 terminus is unimportant for pigment binding, we deduce that a 9 amino acid segment (204–212) at the COOH terminus which includes His208 (one of the three highly conserved His residues in LHCP II proteins) is essential. Deletion of 15 or 16 amino acids (including 3 Asn residues and 1 Trp residue outside the putative amphipathic helix) from the COOH terminus apparently results in a protein that can be only partially reconstituted. The Asn residues are present in most Cab proteins. Analyses of fluorescence and absorption spectra of the partially reconstituted COOH-terminal mutant constructs indicate disconnection of a fraction of the bound Chl *b* and a diminished Chl *a*/*b* ratio (to 0.8). Nonetheless, all of the major pigment species are present and only the negative 683-nm CD signal is absent. Constructs with differing 3′ vector-encoded amino acids fused to similar AB96 deletion mutant proteins (data not shown) provide evidence against the possibility of destabilizing effects from artifacts generated by the *E. coli* expression system. Because of the low yield of reconstituted CP2 from these mutant proteins following electrophoresis, increased lability of the complexes probably results from the deletion mutations.

The NH_2-terminal deletion construct lacking 49 amino acids may also result in a protein that can undergo only partial reconstitution. Most of the conserved β-turn region between helix B and the NH_2 terminus is absent from this product. Although the biochemical evidence and spectroscopic evidence suggest that a nearly complete or normal CP2 complex can be reconstituted from this mutant protein, we observe variability in yield, degree of connectedness of Chl *b* to Chl *a*, and the intensity of the negative 683-nm CD signal from experiment to experiment. Again, stability of reconstituted complexes during electrophoresis and subsequent handling procedures becomes an important consideration. It is possible that the specific pigment organization reflected in the 683-nm CD signal is formed during the freeze/thaw reconstitution process but is subsequently destroyed during LDS–PAGE. A number of consistent observations lead us to favor this hypothesis: (1) the 683-nm CD signal is more prominent in TX100-derived LHC than in electrophoretically-isolated native CP2; (2) this signal was not consistently observed during initial reconstitution studies (Plumley & Schmidt, 1987; Cammarata & Schmidt, 1990) but is now routinely obtained with reconstituted CP2 [see also Paulsen et al. (1990)] due to shorter electrophoresis times; (3) violaxanthin is nearly absent from recovered CP2 (native or reconstituted), but it is present in TX100-derived LHC; (4) omission of one or two xanthophyll species (including violaxanthin) from the reconstitution results in an uncoupling of some Chl *b* from Chl *a* (Plumley & Schmidt, 1987); (5) the Chl *a*/*b* ratio observed for native CP2 and especially reconstituted CP2 is both decreased and variable with respect to that of TX100-derived LHC. Despite these qualifications, the stoichiometries of Chl *b*, xanthophylls, and apoproteins in reconstituted samples closely resemble those of native complexes. Thus, the absence of the negative 683-nm CD signal observed for some of the reconstituted AB96 COOH-terminal deletion mutant gene products is best explained by dissociation of some Chl *a* during PAGE due to a loss of amino acids which stabilize pigment–protein interactions.

The definitive test for functional reconstitution of LHCP II is to demonstrate excitation energy transfer to a photosystem II reaction center in vitro. Such transfer has been well-documented for TX100-derived LHC and appears to require diacylglycerides (Sprague, 1987). However, to our knowledge, excitation transfer to a reaction center complex has not been demonstrated for CP2 isolated by PAGE and attempts using native and reconstituted CP2 so far have not been successful in our laboratory. More complete characterization of the composition and energy transfer properties of reconstituted LHC will require the development of alternative recovery methods to overcome the limitations imposed by LDS–PAGE.

In conclusion, our data support the structural assignments discussed above and show directly that some of the surface-exposed amino acids affect pigment binding. Loss of 8 amino acids (50–57) from the stroma-exposed charged portion of helix B has a pleiotropic effect on pigment binding/stability as does removal of 9 amino acids (204–212) including His208 from the COOH-terminal amphipathic portion of helix A with lumenal exposure. If regions of structural importance for pigment-binding stability reside in α-helical regions, then our results delineate these helices at the vicinities of residues 49 and 213, respectively, on the NH_2- and COOH-terminal surfaces. Residues 213–218 are correlated with stability of some Chl *a* which is susceptible to dissociation during LDS–PAGE. Importantly, we show that 26% of an LHC apoprotein is not involved in pigment binding. We can now rationally pursue site-directed mutagenesis of the residues that remain as candidates for pigment binding.

Acknowledgments

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Reaction of Ferrous Cytochrome *c* Peroxidase with Dioxygen: Site-Directed Mutagenesis Provides Evidence for Rapid Reduction of Dioxygen by Intramolecular Electron Transfer from the Compound I Radical Site†

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ABSTRACT: The reaction of dioxygen with the ferrous forms of the cloned cytochrome *c* peroxidase [CCP(MI)] and mutants of CCP(MI) prepared by site-directed mutagenesis was studied by photolysis of the respective ferrous–CO complexes in the presence of dioxygen. Reaction of ferrous CCP(MI) with dioxygen transiently formed a Fe^{II}–O_2 complex (bimolecular rate constant = $(3.8 \pm 0.3) \times 10^4$ M^{-1} s^{-1} at pH 6.0; 23 °C) that reacted further (first-order rate constant = 4 ± 1 s^{-1}) to form a product with an absorption spectrum and an EPR radical signal at $g = 2.00$ that were identical to those of compound I formed by the reaction of $CCP(MI)^{III}$ with peroxide. Thus, the product of the reaction of $CCP(MI)^{II}$ with dioxygen retained three of the four oxidizing equivalents of dioxygen. Gel electrophoresis of the $CCP(MI)^{II}$ + dioxygen reaction products showed that covalent dimeric and trimeric forms of CCP(MI) were produced by the reaction of $CCP(MI)^{II}$ with dioxygen. Photolysis of the $CCP(MI)^{II}$–CO complex in the presence of ferrous cytochrome *c* prevented the appearance of the cross-linked forms and resulted in the oxidation of 3 mol of cytochrome *c*/mol of $CCP(MI)^{II}$–CO added. The results provide evidence that reaction of $CCP(MI)^{II}$ with dioxygen causes transient oxidation of the enzyme by 1 equiv above the normal compound I oxidation state. Mutations that eliminate the broad EPR signal at $g = 2.00$ characteristic of the compound I radical also prevented the rapid oxidation of the ferrous enzyme by dioxygen. The Trp 191 → Phe, Gln, His and Asp 235 → Asn mutants readily formed a ferrous–dioxygen complex, but the rapid further reaction of this intermediate to an oxyferryl form was not observed. Instead, the Fe^{II}–O_2 enzymes returned slowly to the ferric state without detectable accumulation of intermediates (apparent rate constant = $(1 \pm 0.5) \times 10^{-4}$ s^{-1}). Other active site mutations did not prevent the rapid oxidation of the ferrous enzyme to compound I by dioxygen. The dramatic effects of mutations at Trp 191 and Asp 235 are interpreted as an indication that intramolecular electron transfer from the compound I radical site to the coordinated dioxygen ligand promotes the rapid oxidation of $CCP(MI)^{II}$ by dioxygen.

Cytochrome *c* peroxidase (cytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5; CCP[1]) is a monomeric heme protein that catalyzes the peroxide-dependent oxidation of ferrous cytochrome *c*. Although the normal catalytic cycle of CCP involves iron in the ferric and higher oxidation states, the enzyme can be reduced to the ferrous state under anaerobic conditions. The reactivity of ferrous CCP with dioxygen differs dramatically from that of other heme peroxidases. Photolysis of CCP^{II}–CO in the presence of dioxygen rapidly converts the enzyme to the oxyferryl state (Wittenberg et al., 1968). This product has been reported to be identical to compound I (or compound ES) produced by the reaction of the ferric enzyme with peroxide (Anni et al., 1985). In contrast, other ferrous

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[1] Abbreviations: CCP, cytochrome *c* peroxidase from bakers' yeast; CCP(MI), a cloned cytochrome *c* peroxidase expressed in *E. coli*; HRP, horseradish peroxidase; cyt *c*, cytochrome *c*; oxidation states of the respective enzymes are indicated by superscripts, i.e., $CCP(MI)^{II}$, ferrous CCP(MI); $trp^{\bullet}_{191}$; 1-N indolyl radical at Trp 191 of CCP; $por^{\bullet+}$, porphyrin π-cation radical; compound I, $CCP(MI)^{IV}{=}O(trp^{\bullet}_{191})$, the two-electron oxidation product of the reaction of ferric CCP(MI) with peroxide; compound I′, the product of $CCP(MI)^{II}$ oxidation by dioxygen; compound II_R, $CCP^{III}(trp^{\bullet}_{191})$, the transient product obtained by one-electron reduction of compound I by cyt c^{II}; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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