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In Vitro Reconstitution of the Recombinant Photosystem II Light-harvesting Complex CP24 and Its Spectroscopic Characterization*

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The light-harvesting chlorophyll *a/b* protein CP24, a minor subunit of the photosystem II antenna system, is a major violaxanthin-binding protein involved in the regulation of excited state concentration of chlorophyll *a*. This subunit is poorly characterized due to the difficulty in isolation and instability during purification procedures. We have used an alternative approach in order to gain information on the properties of this protein; the *Lhcb6* cDNA has been overexpressed in bacteria in order to obtain the CP24 apoprotein, which was then reconstituted *in vitro* with xanthophylls, chlorophyll *a*, and chlorophyll *b*, yielding a pigment-protein complex with properties essentially identical to the native protein extracted from maize thylakoids. Although all carotenoids were supplied during refolding, the recombinant holoprotein exhibited high selectivity in xanthophyll binding by coordinating violaxanthin and lutein but not neoxanthin or β-carotene. Each monomer bound a total of 10 chlorophyll *a* plus chlorophyll *b* and two xanthophyll molecules. Moreover, the protein could be refolded in the presence of different chlorophyll *a* to chlorophyll *b* ratios for yielding a family of recombinant proteins with different chlorophyll *a/b* ratios but still binding the same total number of porphyrins. A peculiar feature of CP24 was its refolding capability in the absence of lutein, contrary to the case of other homologous proteins, thus showing higher plasticity in xanthophyll binding. These characteristics of CP24 are discussed with respect to its role in binding zeaxanthin in high light stress conditions.

The spectroscopic analysis of a recombinant CP24 complex binding eight chlorophyll *b* molecules and a single chlorophyll *a* molecule by Gaussian deconvolution allowed the identification of four subbands peaking at wavelengths of 638, 645, 653, and 659 nm, which have an increased amplitude with respect to the native complex and therefore identify the chlorophyll *b* absorption in the antenna protein environment. Gaussian subbands at wavelengths 666, 673, 679, and 686 nm are depleted in the high chlorophyll *b* complex, thus suggesting they derive from chlorophyll *a*.

In higher plants, chloroplasts, chlorophyll, and carotenoid molecules are noncovalently bound to specific transmembrane proteins to form light-harvesting complexes called LHCI[1] and LHCII. These antenna complexes efficiently capture the light and deliver the excitation energy, respectively, to photosystem I (PSI) and II (PSII) reaction centers, where electron transport occurs, yielding a *trans*-thylakoid pH gradient, ATP synthesis, and $NADP^+$ reduction. The photosystem II light-harvesting complex has been extensively investigated and shown to be composed of four chlorophyll *a*/*b* proteins, the major complex (LHCII) binding about 65% of PSII chlorophyll and three minor complexes (called CP24, CP26, and CP29) that together bind about 15% of total PSII chlorophyll (1). These minor chlorophyll proteins appear to be involved in the dissipation of the chlorophyll excitation energy needed to prevent overexcitation and photoinhibition of PS II (see Ref. 2 for a review). It was shown that more than 80% of the xanthophyll violaxanthin is associated to minor complexes in maize (3) and that CP24 is the one with the highest violaxanthin binding capacity. This pigment is involved in the major photoprotection mechanism in plants, known as "nonphotochemical quenching" (4), through the operation of a xanthophyll cycle by which it is deepoxidated to antheraxanthin and zeaxanthin (5). The intermediate location of minor complexes between the reaction center and the major LHCII complex (6) makes them well suited for regulating the excitation energy supply to PSII or its dissipation. The structural bases for the regulatory properties of the minor chlorophyll proteins are mostly unknown due to the difficulties in the isolation of these proteins in sufficient amounts and in their native form. To overcome this problem, we have reconstituted the CP24 holoprotein using overexpressed apoprotein from *Escherichia coli* and purified pigments. Its characterization allowed us to obtain previously unavailable information on the number of chromophores bound to this protein and opens the way to the mutational analysis of this PSII subunit in both its protein moiety and the chromophores bound. As an example of the usefulness of recombinant pigment-proteins, we have used the recombinant CP24 in order to address the problem of Chl *a* and Chl *b* absorption in antenna proteins; while only two chemically distinct chlorophyll species are present, many optical transitions (spectral forms) are commonly observed in the Qy absorption region (7–12). Lack of progress in understanding this spectroscopic heterogeneity has been mainly due to the absence of experimental techniques to enable selective modification of the optical transitions. Also, it has not been possible to

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[1] The abbreviations used are: LHCI, light-harvesting complex of PSI; LHCII, light-harvesting complex of PSII; Chl, chlorophyll; CP, chlorophyll protein; DM, dodecylmaltoside; FWHM, full-width half-maximum; LiDS, lithium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PSI, photosystem I; PSII, photosystem II; rCP24 and rCP29, recombinant CP24 and CP29, respectively, reconstituted from the apoprotein derivatives overproduced in bacteria; nCP24, native CP24; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography.

assign particular transitions to Chl *a* or Chl *b* though it is generally assumed that the shorter wavelength bands are associated with Chl *b*. Analysis of a recombinant CP24 complex binding mainly Chl *b* allowed us to identify four Gaussian subbands peaking at 638, 645, 652, and 659 nm, which are also present in the native complex, thus identifying the principle components of Chl *b* absorption in the antenna protein environment.

EXPERIMENTAL PROCEDURES

Construction of a CP24 (Lhcb6) Expression Plasmid—To overexpress plant CP24 in *E. coli*, the maize *Lhcb6* cDNA (13) was subcloned into an expression vector of the pDS series (14). A clone was obtained by polymerase chain reaction mutagenesis of *Lhcb6* DNA. The construct pDS 12–24$\alpha\epsilon$ (Fig. 1*A*) was obtained by using two primers (5′-CCGCGCGCAGATCTTCGCC-3′ (carrying the *Bgl*II site) and 5′-TCTGATCCCATGCATCCGTACGTC-3′ (carrying the *Nsi*I site)), allowing the amplification of a 733-base pair fragment spanning the full coding region. After digestion with *Bgl*II and *Nsi*I, the resulting fragment was subcloned into the pDS-RBS II expression vector. Thus, the pDS 12–24$\alpha\epsilon$ construct codes for a protein containing four additional residues (MRIA . . .) extending the N teminus as compared with the native protein (Fig. 1*B*). Plasmids were constructed using a standard molecular cloning procedures (15). Bacterial hosts were *E. coli* (SG13009 strain) (16).

Isolation of Overexpressed CP24 Apoprotein from Bacteria—CP24 apoprotein was isolated from the SG13009 strain transformed with the construct according to the protocols in Refs. 17 and 18.

Pigment Purification and Analysis—Total pigment extracts were obtained by extracting thylakoids of wild-type barley with 80% acetone. Extracts of Chl *a* and carotenoids were obtained by using thylakoids from the Chl *b*-less mutant *chlorina f2* (19). Chl *b* and carotenoids were obtained by preparative HPLC using a reverse phase column (PHENOMENEX, Torrance, CA) bondclone 10 C18 (7.8 × 300 mm) using 82% acetone as eluent. Reconstituted complexes were analyzed for their pigment composition after 80% acetone extraction as described previously (3). During all of the procedures, care was taken to protect pigments from light and oxygen. The concentration of pigments was determined spectroscopically according to Ref. 20 for chlorophylls and using the extinction coefficients given by Davies (21) for xanthophylls. The concentration of carotenoid mixtures was estimated on the basis of an average molar extinction coefficient of 1.4×10^{-5} at 444 nm. Pigment composition of chlorophyll proteins was determined by HPLC analysis according to Ref. 22. Chl *a*/*b* ratio and Chl/carotenoid ratio was also determined by fitting the spectrum of ethanol extracts with the spectra of purified pigments.

Reconstitution of CP24-Pigment Complexes—The reconstitution procedure largely followed the one designed for LHCII (18, 23). In the basic procedure, 400 μg of protein of CP24 apoprotein isolated from bacteria was solubilized in 1000 μl of a buffer containing 100 mM Tris-HCl (pH 10), the protease inhibitors 6-aminocaproic acid (5 mM) and benzamidine (1 mM), 12.5% sucrose, 2% LiDS by heating to 100 °C for 3 min and sonication. After the addition of 100 mM dithiothreitol and the pigment solution in 70 μl of ethanol, the mixture was sonicated again. Reconstitution was achieved by three subsequent cycles of freezing (1 h, −20 °C) and thawing (30 min, room temperature). Octyl β-D-glucopyranoside was then substituted for LiDS by precipitation of the potassium dodecyl sulfate following the addition of 4% KCl, incubation for 15 min in ice, and centrifugation (10 min at 13,000 × *g*). The mixture was then loaded on a 12-ml sucrose gradient (0.1–1 M) containing 10 mM Hepes, pH 7.6, and 0.06% DM and centrifuged for 17 h at 254,000 × *g* in a Beckman SW 41 rotor. The lower green band (at about 0.4 M sucrose) contained the reconstituted complex and was harvested with a syringe. Maximal yield was obtained with chlorophyll:protein molar ratios between 40 and 80, corresponding to a 5–10-fold excess of pigments. In this work, the carotenoid concentration was maintained at 60 μg/ml.

Removal of Excess Pigments from the Reconstituted Complex—The green bands from the sucrose gradient were then subjected to chromatography on a Fractogel EMD-DMAE column (15 × 150 mm) (Merck). After loading, the column was washed with 0.025% DM (70 min at 1 ml/min). The chlorophyll-protein was then eluted by applying a 0–500 mM NaCl gradient. The peak fractions were concentrated by Centricon centrifugation and loaded into a glycerol gradient (10–25%) containing 0.06% DM and 10 mM Hepes, pH 7.6. The gradient was spun overnight in SW 60 Beckman rotor at 450,000 × *g*, yielding a faint upper band of free pigments and a lower band with the chlorophyll-protein, which was frozen in liquid nitrogen and kept at −80 °C until use.

Isolation of Native CP24—CP24 was isolated from maize PSII membranes as described previously (1, 24).

Electrophoresis—Mildly denaturing electrophoresis was according to Ref. 25 but at 4 °C and with 20% glycerol in resolving and stacking gel. Denaturing electrophoresis was according to Ref. 26. Protein concentration was determined by the bicinchoninic acid method (27).

Densitometry—Densitometry was performed with a Bio-Rad 600 scanning densitometer after staining of the gel and destaining according to Ref. 28.

Spectroscopy—Absorption spectra were obtained using an SLM-Aminco DW-2000 spectrophotometer at room temperature. Fluorescence excitation and emission spectra were obtained by using a Jasco-600 spectrofluorimeter. Samples were in 10 mM Hepes, pH 7.6, 0.06% DM, 20% glycerol. Chlorophyll concentration was about 10 μg/ml for absorption measurements and 0.01 μg/ml for fluorescence measurements. Emission and excitation spectra were corrected for instrumental response. Analysis of fluorescence spectra was performed according to Stepanov as previously reported (12, 29). Circular dichroism spectra were recorded with a Jasco J-600 spectropolarimeter as previously reported (12).

Gaussian Decomposition of Absorption Spectra—Similarly to a described method (11), the decomposition of the absorbance *versus* wavelength was obtained by a nonlinear least squares fitting code (Origin™; MicroCal. Software Inc., Northampton, MA). Here, a linear combination of maximum 10 symmetric Gaussians (eight absorption bands plus two border ones for tails' adjustment) was considered in the χ^2 minimization (error considered by counting statistics) by means of the Levenberg–Marquardt algorithm (30). All of the parameters were kept free and always unconstrained in the analysis; the fitting procedure was reproducible when starting from a reasonable initial choice for subband FWHM (less than 10 nm shared by all subbands) and peaks (within the 630–720 wavelength range). Less than 50 iterations were necessary for achieving the subband positioning, and, after assigning the FWHM to the different Gaussians, the convergence was estimated by a χ^2 variation down to a few percentages in successive single iterations.

RESULTS

Expression of Maize CP24 Gene Construct in Bacteria and Isolation of Overexpressed Apoprotein from Bacteria—The *E. coli* host cells SG13009 (16) are a K12-derived strain. They were transformed with the pREP4 plasmid, which carries the kanamycin selection and the *lacI* gene, encoding the Lac repressor, thus allowing a tight control over the level of expression (Qiagen). The construct is described in Fig. 1*A*, and the sequence coded is shown in Fig. 1*B*. The bacterial strain transformed with the pDS 12–24$\alpha\epsilon$ produced, upon induction with 2 mM isopropyl-1-thio-β-D-galactopyranoside, the protein of the expected molecular weight as detected by Western blotting with a CP24 antibody (31) (Fig. 1, *C* and *D*). The best results were obtained after 6–7 h of isopropyl-1-thio-β-D-galactopyranoside induction in superbroth. In all conditions tested, the expressed protein reached 10% of the total protein extract.

Fractionation of the bacterial cells by the method in Ref. 17 showed that the expression products are accumulated in inclusion bodies, as shown for LHCII (18, 32) and CP29 (33). Repeated washings of this pelletable fraction yielded 80% pure CP24 apoprotein as judged by polyacrylamide electrophoresis. The protein could be easily purified to homogeneity by preparative isoelectric focusing, but most of the experiments here described were performed with the 80% pure preparation without affecting the efficiency of the reconstitution. All experiments described in this study have been performed with rCP24 purified as shown in Fig. 1*C*.

Reconstitution of Pigment-containing Complexes—Triton X-100-washed inclusion bodies (15) were extracted with 80% acetone in order to remove residual Triton and solubilized in 2% LiDS in a bath sonicator followed by boiling. Pigments were then added from stock solutions in ethanol, and the reconstitution procedure was carried out as described under "Experimental Procedures." In this experiment, a Chl *a* to Chl *b* ratio

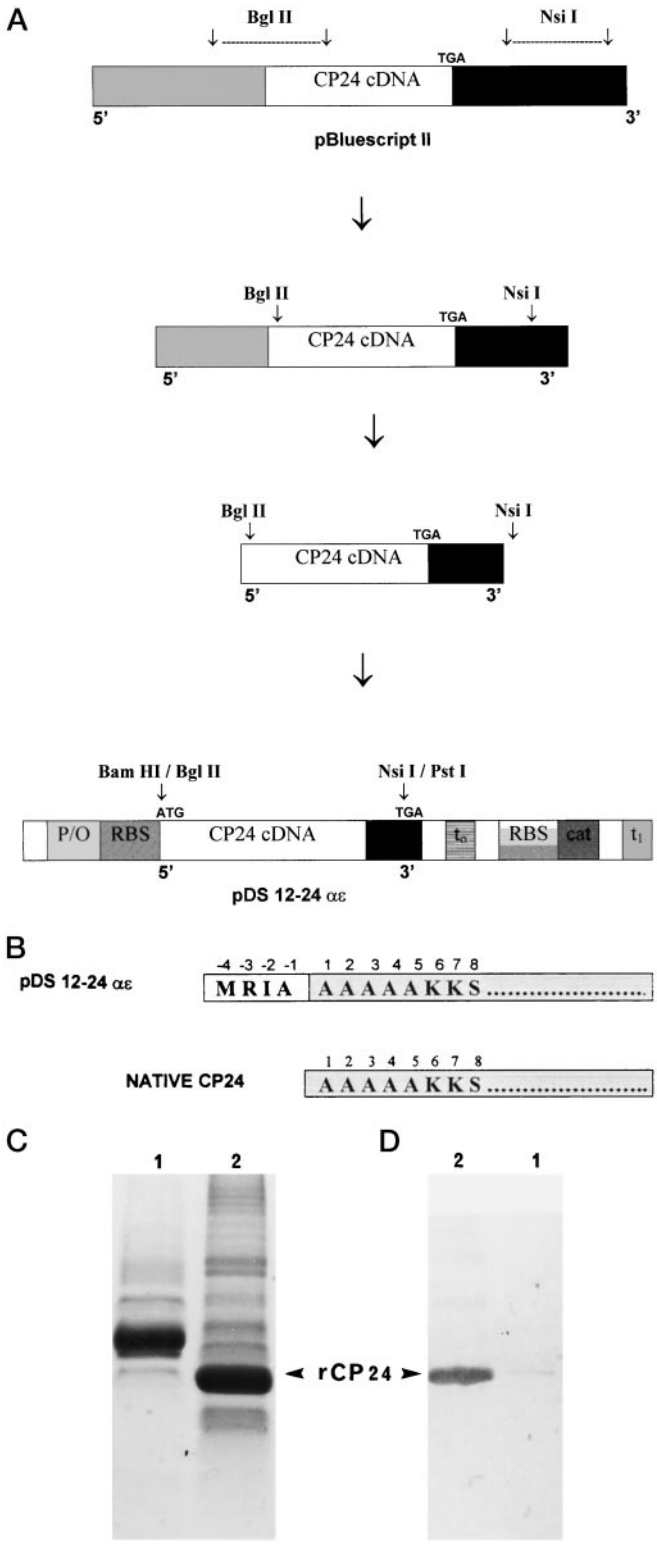

FIG. 1. **Expression of rCP24 by *E. coli* containing *Lhcb6* expression plasmid and isolation of rCP24 apoprotein.** *A*, construction of CP24-expression plasmid. *B*, N-terminal sequences of the native CP24 protein and of the recombinant proteins used in this study as deduced from DNA sequencing. The first residue of the mature protein is labeled *1*; residues labeled with negative numbers derive from the transit peptide; *C*, Coomassie stained SDS-PAGE; *D*, immunoblotting with antibody directed to CP24. *Lane 1*, BBY PSII particles; *lane 2*, inclusion body preparation.

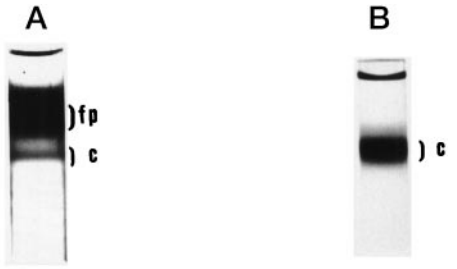

FIG. 2. **Isolation of reconstituted CP24 complex.** *A*, sucrose gradient ultracentrifugation; *B*, glycerol gradient ultracentrifugation. The lower band from the gradient in A was subjected to ion exchange chromatography (see "Experimental Procedures"), and the eluate was spun into a glycerol gradient. A single green band was detected, containing the protein while the upper, free pigment containing band was absent.

of 1.6, similar to the ratio in the native complex (1), was used, and the mixture contained a total carotenoid extract from thylakoids, thus including β-carotene, lutein, neoxanthin, violaxanthin. Following reconstitution, the mixture was ultracentrifuged through a 0.1–1 M sucrose gradient, yielding a green band at 0.4 M sucrose, well separated from the free pigment band on the upper part of the tube at 0.1–0.2 M sucrose (Fig. 2*A*). The lower green band was harvested with a syringe and subjected to DMAE chromatography. Once bound to the column in 10 mM Hepes-KOH, pH 7.6, 50 mM NaCl, 0.03% DM, the nonspecifically bound pigments were washed from the complexes and the column with 10 mM Hepes-KOH, pH 7.6, 50 mM NaCl, 0.03% DM at 1 ml/min. This procedure removed about 50% of the chlorophyll loaded in the column but not the protein. The protein was eluted at 200 mM NaCl. When centrifuged on a 10–25% glycerol gradient, the eluted protein appeared as a single green band without residual free pigments (Fig. 2*B*).

Influence of the Xanthophyll Availability on Reconstitution—When isolated from thylakoid membranes, CP24 binds lutein and violaxanthin but not neoxanthin (3). In order to elucidate the role of different xanthophyll species in refolding of CP24, we have carried on the reconstitution procedure with pigment mixtures differing in xanthophyll composition. In each experiment, one xanthophyll species was omitted from the mixture. Alternatively, reconstitution without xanthophylls (only Chl *a* and Chl *b*) or with the complete carotenoid set was performed. The stability of the resulting complex was assayed at two levels of stringency: sucrose gradient ultracentrifugation (Fig. 3*A*) and mildly denaturing LiDS-PAGE (Fig. 3*B*). When xanthophylls were omitted from the mixture, a green band was still obtained after sucrose gradient ultracentrifugation, having the same mobility as the control sample with a complete xanthophyll supply, suggesting a partial folding was obtained even in the absence of carotenoids. However, this complex did not survive LiDS-PAGE. The samples refolded in the presence of two xanthophylls yielded a green band stable both in sucrose gradients and in LiDS-PAGE, and the yields were comparable with each other and with the control sample prepared in the presence of the three xanthophylls. Particularly interesting is the case of sample A, where a stable complex was obtained in the absence of lutein. This result is at variance with previous results with LHCII and CP29 (18, 33) in which lutein was shown to be essential for stability.

Influence of Chlorophyll a to Chlorophyll b Ratio during Refolding on the Pigment Binding Properties of Recombinant CP24—The data reported above show that CP24 can be successfully refolded *in vitro*. The use of this recombinant protein for mutation analysis is, however, more informative if its characteristics closely reflect those of the native complex extracted from thylakoid membranes. We optimized the conditions for the recombinant protein to reproduce the chlorophyll *a*/*b* ratio of native CP24. For comparison, native CP24 was purified from maize PSII membranes as described previously (1), and it

showed an a/b ratio of 1.2. A series of reconstitution mixtures were performed with decreasing Chl a/b ratios, namely 8.0, 5.8, 2.0, 1.5, 1.0 and 0.001. The last preparation was intended to contain only Chl *b*. However, HPLC analysis of the reconstitution mixture showed 1:1000 contamination by Chl *a*. This experiment was performed on a semipreparative scale, following the procedure in Fig. 2, finally obtaining, from glycerol gradient ultracentrifugation, sufficient amounts of the complex for spectroscopic and biochemical analysis. In Fig. 4, a plot is shown of the dependence of the Chl a/b ratio in the complex on the Chl a/b ratio in the reconstitution mixture; although the reconstitution with Chl *b* only yielded a stable complex having a Chl a/b ratio of 0.12, the Chl a/b ratio of the reconstituted complex rises steeply to about 1 with increasing Chl *a* availability and reaches a plateau between ratios of 2.0 and 5.8. Within this range, a ratio of 1.0 for bound Chl *a* and Chl *b* was obtained. When a large Chl *a* excess was applied (Chl a/b ratio of 8) it was possible to obtain a Chl a/b ratio in the reconstituted complex of 1.4. As a comparison, the data previously obtained for CP29 (12, 33) are also reported, showing that the two proteins clearly differed in their affinity for Chl *a* and b. From this comparison, the two complexes with Chl a/b of 1.0 and 1.4 most closely resemble native CP24.

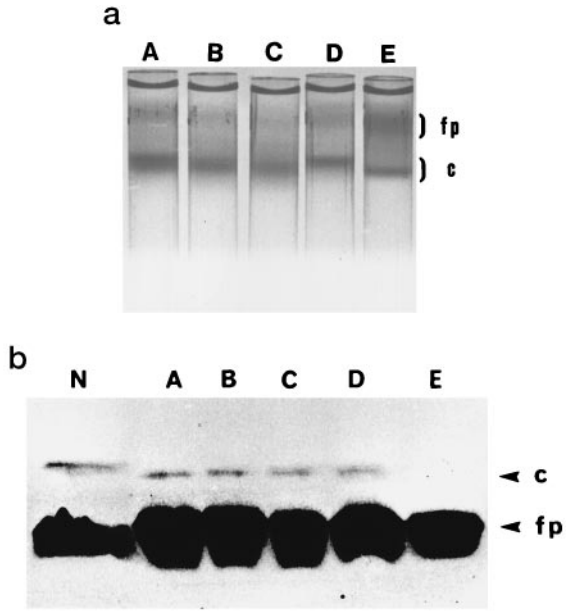

FIG. 3. **Influence of the carotenoid composition on reconstitution of recombinant CP24.** *a*, sucrose gradient ultracentrifugation; *b*, LiDS-PAGE**.** The gel was not stained. The refolding mixture contained the following pigments: Chl *a* + Chl *b* + violaxanthin + neoxanthin (lutein missing) (*A*); Chl *a* + Chl *b* + lutein + neoxanthin (violaxanthin missing) (*B*); Chl *a* + Chl *b* + violaxanthin + lutein (neoxanthin missing) (*C*); Chl *a* + Chl *b* + violaxanthin + lutein + neoxanthin (total xanthophylls) (*D*); Chl *a* + Chl *b* (without xanthophylls) (*E*); native CP24 (*N*).

Biochemical Characterization of Recombinant CP24—In order to further characterize the recombinant CP24 complex (a/b = 1.4 and a/b = 1.0), we determined the chlorophyll to protein stoichiometry. Toward this end, we used a highly purified LHCII preparation (24) as a standard protein binding 12.6 ± 0.1 chlorophylls/polypeptide (1, 34). The chlorophyll concentration of the LHCII and of CP24 samples was carefully determined by HPLC analysis, and aliquots of the samples corresponding to different amounts of chlorophyll were loaded on an SDS-PAGE gel. After running, the gel was stained for quantitative analysis according to Ball (28), and the Coomassie Blue binding to CP24 and LHCII gel bands was determined by densitometry and by elution of stain and spectrophotometric determination. The Coomassie Blue-stained gel and the resulting plot is shown in Fig. 5, *B* and *C*. Pigment binding to rCP24 was 0.79 with respect to LHCII. Once corrected for the sequence-specific difference in Coomassie Blue binding between CP24 and LHCII (1), a value of 10 chlorophyll (*a* plus *b*) mol per mol of CP24 apoprotein was obtained.

The results of HPLC analysis of pigments extracted from native and recombinant CP29 proteins are reported in Table I. Both the native and the recombinant protein contained, besides Chl *a* and *b*, the xanthophylls lutein and violaxanthin but not neoxanthin and β-carotene, although the latter carotenoids were present in the reconstitution mixture in the same amounts as lutein and violaxanthin. Per 10 chlorophylls (*a* plus *b*), two xanthophylls were found per rCP24 polypeptide. This is consistent with the value of two xanthophyll molecules per polypeptide found in the homologous protein CP29 (35). Table I also reports HPLC analysis of the rCP24 proteins with Chl a/b ratio of 0.12, obtained by refolding with Chl a/b = 0.001. It is shown that the specificity of carotenoid binding is retained irrespective of the binding of Chl *a* or Chl *b*, since neoxanthin was never present in the proteins. If a value of two xanthophyll molecules per polypeptide is assumed (as in the rCP24 1.4 and

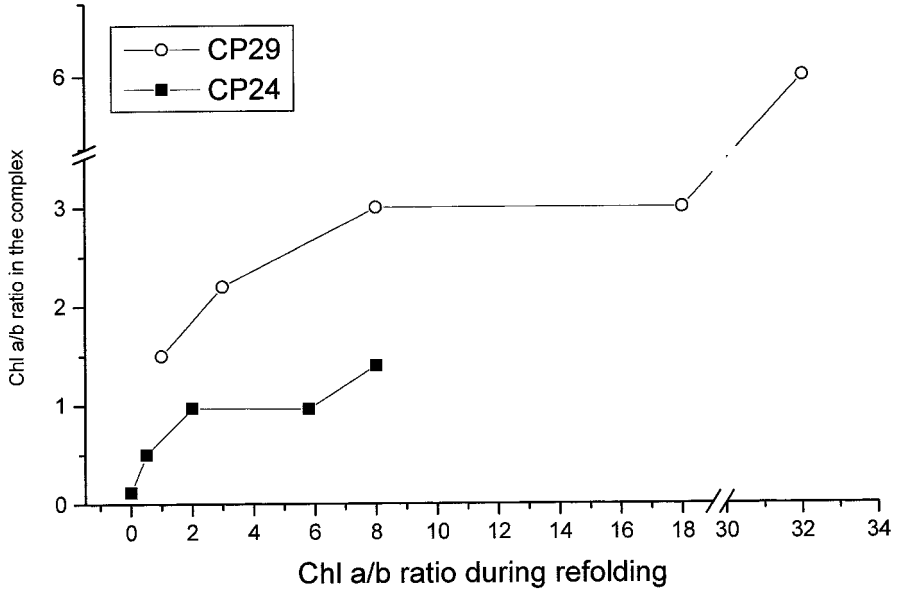

FIG. 4. **Plot of the dependence of the Chl *a/b* ratio in rCP24 on the Chl *a/b* ratio of the reconstitution mixture during refolding.** In this experiment, all carotenoids were present in excess.

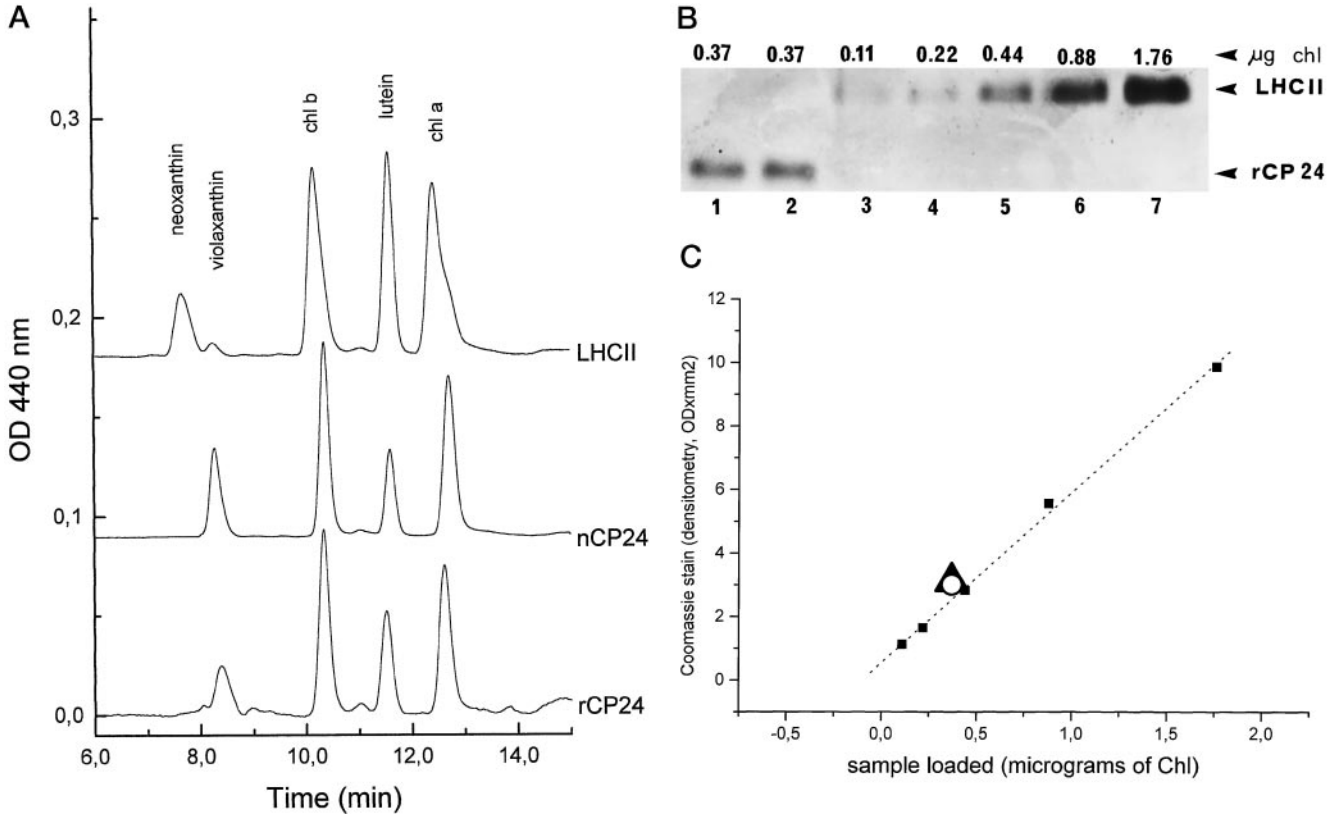

FIG. 5. **Pigment binding properties of rCP24.** *A*, HPLC analysis of rCP24 1.0 and native CP24 with native LHCII as a reference. Note the absence of a neoxanthin peak in rCP24 and nCP24. *B*, SDS-PAGE analysis of the protein:chlorophyll ratio. LHCII, used as a reference, was loaded in different amounts in *lanes 3–7*. *Numbers above* refer to the amount of chlorophyll loaded on the individual lanes. rCP24 1.4 and rCP24 1.0 (0.37 µg of Chl *a* + Chl *b*) were loaded respectively on *lanes 1* and *2*. After running, the gel was stained with Coomassie Blue. *C*, plot of the data obtained by densitometry of the gel of *B*. ■, LHCII; ▲, rCP24 1.0 (0.37 µg of Chl); ○, rCP24 1.4 (0.37 µg of Chl).

TABLE I
HPLC analyses of nCP24, rCP24 1.0, rCP24 1.4, rCP24 0.12, and LHCII

Values are in mol/100 mol of Chl *a*. Data are the average from three replicates.

Sample	Chl *a*/*b* ratio (during folding)	Chl *a*/*b* ratio (in the complex)	Violaxanthin	Lutein	Neoxanthin	Chlorophyll *b*	Chlorophyll *a*
nCP24		1.22	17.5	19.8	0.06	83.4	100
rCP24 1.0	2.0	0.96	11.3	29.7	0.05	103.8	100
rCP24 1.0	5.8	0.95	12.5	27.6	0.06	104.7	100
rCP24 1.4	8.0	1.42	11.8	22.1	0.09	70.7	100
rCP24 0.12	0.001	0.12	79.6	120.4	0.09	800	100
Native LHCII		1.45	1.6	25.7	11.9	71.5	100

TABLE II
Stoichiometry of pigment binding to nCP24, recombinant CP24 1.0, recombinant CP24 1.4, recombinant CP24 0.12, and native LHCII

Sample	Chl *a* + *b*/Polypeptide	Xanthophylls/Polypeptide	Chl *a*/Polypeptide	Chl *b*/Polypeptide
nCP24	5[a]	1	2.7	2.3
rCP24 1.0	10	2	5	5
rCP24 1.0	10	2	5	5
rCP24 1.4	10	2	5.8	4.2
rCP24 0.12	9	2	1	8
Native LHCII	12.6[a]	2.7	7.4	5.2

[a] Data are calculated from Table I on the basis of Chl *a* plus Chl *b* to polypeptide stoichiometry measured for native proteins (1) and for recombinant proteins (this work).

rCP24 1.0), then rCP24 0.12 binds one Chl *a* and eight Chl *b* as shown by the Chl:xanthophyll ratio, which is 4.5, thus speaking for nine bound Chl rather than 10. These data are summarized in Table II.

Spectroscopic Characterization of Recombinant CP24—The absorption spectrum of native CP24, which is significantly different with respect to other antenna complexes (10), is shown in Fig. 6*A*. It is characterized by a 675-nm red absorption peak and a 654-nm shoulder. Of the absorption spectra for rCP24 0.12, rCP24 1.0, and rCP24 1.4, only the last two closely resemble nCP24; both the 675-nm peak and the amplitude of the 654-nm shoulder were reproduced in the recombinant protein, while the ratio of the absorption in the Soret region *versus* that in the Qy transition is the same, *i.e.* indicating a similar binding of xanthophylls, relative to Chl, in native and recombinant proteins (see above). The absorption spectrum of rCP24 0.12 was very different; the 675-nm peak disappeared, while the 654-nm form becomes the absolute maximum in the Qy

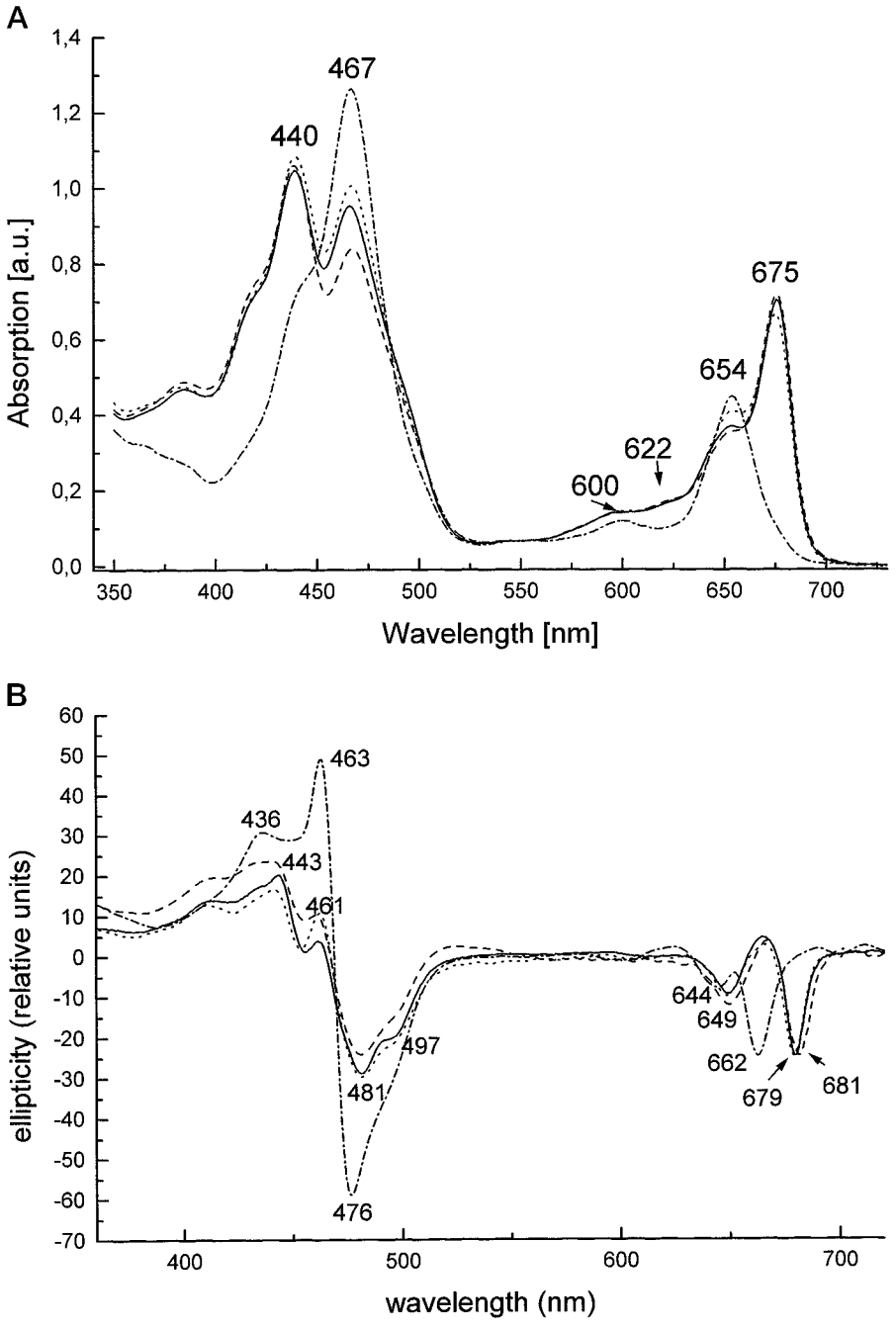

FIG. 6. **Optical spectroscopy of nCP24 and recombinant complexes with different *a/b* ratio.** *A*, absorption spectra. Optical pathlength was 1.0 cm. *B*, circular dichroism. ——, native; – – –, rCP24 1.0; · · · ·, rCP24 1.4; ·–·–·, rCP24 0.12. The samples were on Hepes-KOH, pH 7.6, 0.06% DM, 15% glycerol.

absorption region. Two broader absorption features with low amplitudes were evident between 590 and 630 nm in the spectrum of nCP24, rCP24 1.0, and rCP24 1.4 (*arrows* in Fig. 6*A*). Of these, the red-most one (centered at 622 nm) disappeared in rCP24 0.12. Circular dichroism spectra are shown in Fig. 6*B*. In the case of nCP24, the Qy region is characterized by two negative signals at 679 and 649 nm, while in the Soret region it is negative between 490 and 465 nm with a minimum at 481 nm and a shoulder at 497 nm. Recombinant CP24 had a similar general shape; however, while rCP24 1.0 very closely fitted the nCP24 spectrum, rCP24 1.4 showed a 2-nm red shift in the position of the red-most signal (681 *versus* 679 nm) and in the relative amplitude of the signals in the Soret region. rCP24 0.12 strongly differed in the Qy region, showing a 17-nm blue shift in the red-most band. Moreover, the amplitude of the signal of this sample in the Soret region was much increased and slightly shifted to peaks at 476 nm (−) and 463 nm (+).

Fluorescence spectroscopy was then performed in order to verify the functional connection between pigments bound to the protein in native and recombinant CP29. Fluorescence excitation spectra (with 680-nm emission) are shown in Fig. 7*A*. Chl *a* and Chl *b* contributions are clearly detected at 440 and 465 nm, indicating efficient energy transfer between Chl *b* and Chl *a*. In pigment-proteins, both vibrational relaxation within pigment-excited states and energy transfer between pigments are rapid with respect to the excited state lifetime. Therefore, thermal equilibration between all inter- and intramolecular energy levels is rapidly attained. The actual occurrence of this thermal equilibration can be verified by applying the Stepanov analysis of the steady-state fluorescence (29, 36) by which the fluorescence spectra, expected on the basis of complete thermal equilibration within the complexes, can be calculated from absorption spectra. Measured and calculated spectra will be coincident for fully equilibrated pigment-proteins. When Stepanov analysis was applied to native CP24, quite good correspondence between calculated and measured emission spec-

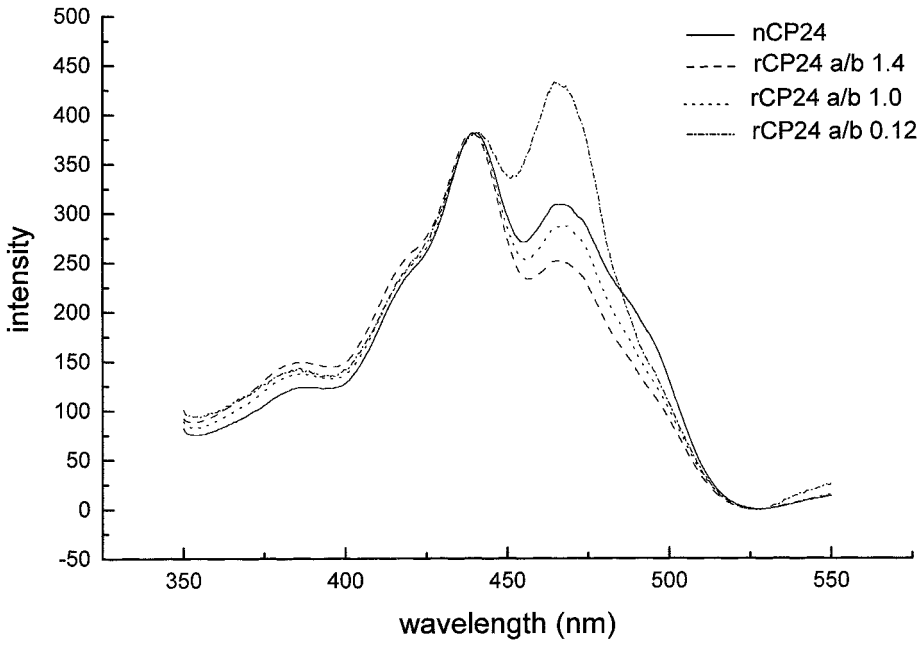

FIG. 7. **Fluorescence excitation spectra of native and recombinant CP24.** Emission was at 681 nm; bandwith for excitation end emission was 2 nm. Sample concentration was less than 0.1 μg of Chl *a* + Chl *b*/ml. Other conditions as in Fig. 6.

tra is observed over most of the emission band (Fig. 8*A*). In the case of rCP24, this correspondence was good for rCP24 1.0 and rCP24 1.4 (Fig. 8, *B* and *C*), while in the case of rCP24 0.12, a distinct deviation was observed (Fig. 8*D*); two emissions were present in both the measured and the calculated spectra at 663 and 679 nm, respectively, corresponding to Chl *b* and Chl *a* emissions as indicated by the relative amplitude of these two components upon 475- and 440-nm excitations. The emission spectrum obtained with 440-nm excitation was similar to the calculated spectrum but showed a higher amplitude of the 663-nm component, thus implying a small excess of excited states on Chl *b*.

Gaussian Deconvolution Analysis of Native and Recombinant CP24—The results reported above show that rCP24 complexes can be obtained *in vitro*, and their chromophore content can be modulated for obtaining a stable complex with altered Chl *a* to Chl *b* ratio. By reconstituting CP24 with a large excess of Chl *b* over Chl *a* (Chl *a*/*b* = 0.001) a CP24 binding eight Chl *b* and one Chl *a* was obtained. Such a complex is ideal for the purpose of addressing the problem of correspondence between chemically distinct chlorophyll species and absorption forms in the protein, since chlorophyll *b* absorption is greater than is the case for proteins extracted from thylakoids and therefore can be analyzed with little interference by Chl *a* absorption. In Fig. 9, the Qy transition region is shown for the absorption spectra, together with the results of Gaussian deconvolution, from native CP24 and three different recombinant complexes (rCP24 0.12, rCP24 1.0, and rCP24 1.4), while the characteristics of this analysis are summarized in Table III. The spectra were deconvoluted as linear combinations of symmetric Gaussians whose parameters (peak wavelength, full-width at half-maximum, and percentage amplitude to the 630–720-nm spectrum integral) were kept free. The only constraint considered was the final control of the total bandwidth in the 9–11-nm range, as expected from the analysis of electron-phonon coupling and site-inhomogeneous broadening in a variety of antenna chlorophyll proteins (10, 12, 37, 38) in the accepted solutions. For the sake of model simplicity and reducing the arbitrariness of the fitting, the minimal choice of adjustable parameters was always used, *i.e.* both the minimal number of Gaussian forms and the curve symmetry for a unique bandwidth; in this sense, at room temperature the absorbance fine structure, like inhomogeneities in bandwidth, are hardly recognizable in the spectra due to the smearing by thermal broadening.

Eight significant Gaussians were needed in order to fit the spectra of the native complex and of the rCP24 1.0 and rCP24 1.4, consistently with the results previously reported for CP29 (12) and LHCII (38). As pointed out above, although the Chl *a*/*b* ratio changed, the total number of Chl molecules bound per CP24 polypeptide remained essentially constant at 10 in the different reconstitution conditions, except for the case of rCP24 0.12, which had an empty site. This implies that several Chl binding sites can either be occupied by Chl *a* or Chl *b*. As can be clearly seen in Table III, the wavelength of the subbands did not change significantly in most of the decompositions. Thus, it should be relatively easy to identify the subbands associated with Chl *b* or with Chl *a* absorption by determining those in which the absorption intensity respectively increases or decreases in the protein with higher Chl *b* binding upon normalization of the spectra. For normalization, we used the measured Chl *a*/*b* stoichiometry assuming a relative Chl *b*/Chl *a* extinction ratio of 0.7, based on solvent values (12, 39). In this respect, the total Qy absorption areas for the different complexes were rescaled to the calculated Chl *a* plus Chl *b* total extinction. The subband amplitudes are then compared with the normalized area and reported for the different complexes in the histogram of Fig. 10 as a ratio of specific subband chloropyll content to total chlorophyll pool. It can be noticed that nCP24, rCP24 1.0, and rCP29 1.4 are rather similar to each other, although rCP24 1.0 most closely fits the values of nCP24. The rCP24 1.4 complex (slightly higher Chl *a* content with respect to nCP24 and rCP24 1.0) had increased absorption at 666 and 673 nm, while the amplitude decrease at 645, 652, and 659 nm suggests that these absorption forms are related to Chl bound to the less selective sites. The case of rCP24 0.12 was the most informative; the 686-nm component was completely absent, and those peaking at 666, 673, and 679 nm were strongly decreased (by 32, 73, and 85%, respectively). On the other hand, the shortest wavelength forms at 638, 645, 652, and 659 nm had their amplitude increased by 13, 35, 54, and 38%, respectively.

As for the blue-most form at 638 nm, all of the complexes, either reconstituted or native, have a chlorophyll ratio of about 1 per site, and (within ±10% of overall error associated with

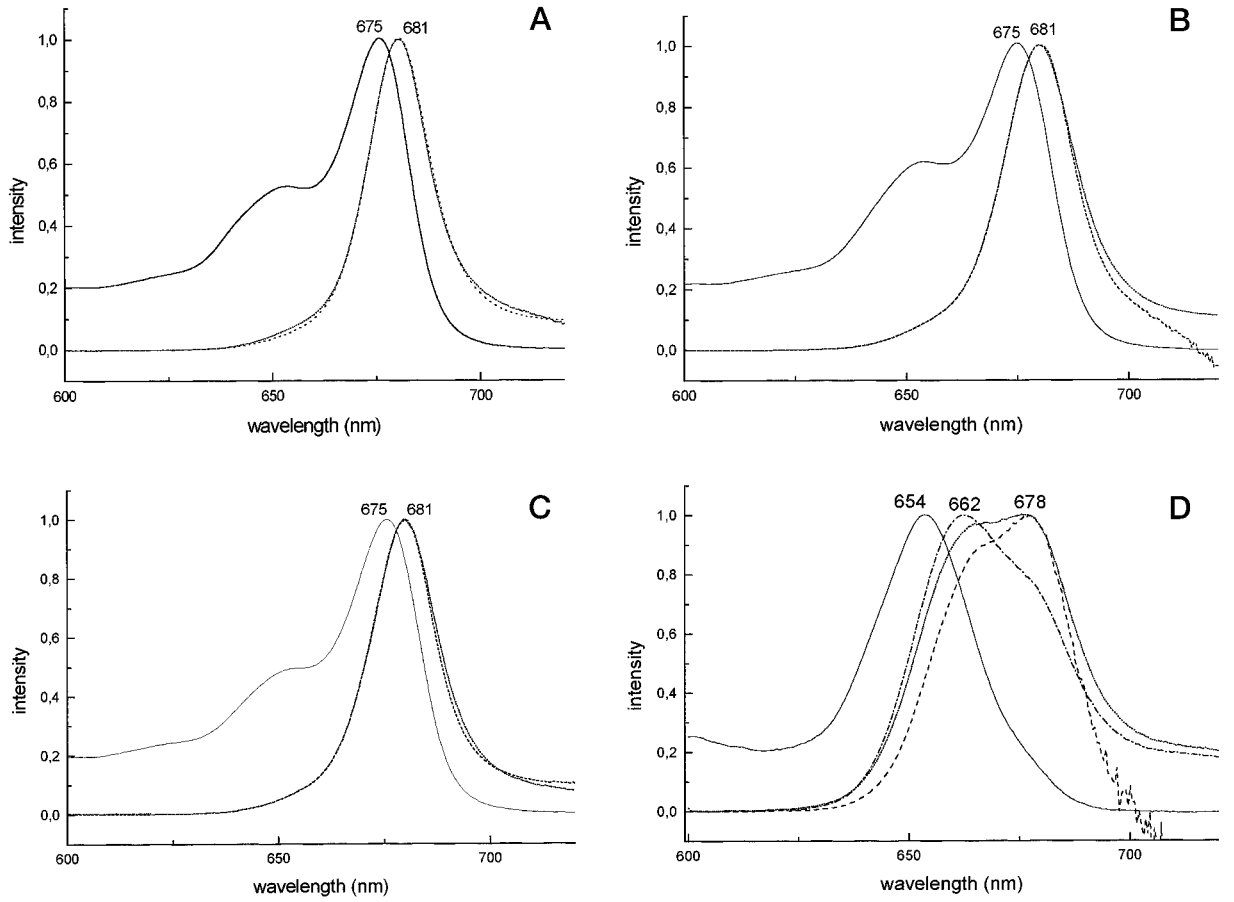

FIG. 8. **Comparison between calculated (*dashed lines*) and measured (*dotted lines*) steady state fluorescence spectra for native and reconstituted CP24 complexes with different Chl *a*/*b* binding ratios.** Emission spectra were calculated from absorption spectra (*solid lines*) using Stepanov's relation (26). *D*, emission spectra with 475-nm excitation (·–·–·) in addition to the 440-nm excitation (*dotted line* in *panels A–D*).

this extrapolation) this ratio suggests the constant filling of this binding site and its unfavored affinity to Chl *a*.

DISCUSSION

The publication of a near atomic structure of LHCII has been a major event in the study of chlorophyll *a*/*b* proteins (34), since this structure gives the first insight into pigment and protein organization not only in the major LHCII complex but also in the homologous proteins that contribute to the organization of the PSI and PSII antenna complexes (40, 41). Nevertheless, a number of questions, important in order to understand the relation between structure and light-harvesting and energy transfer functions of each individual chlorophyll protein and of the whole antenna system need to be addressed. Thus, an assignment of spectroscopic characteristics to the various chromophores in the crystal structure as well as the transition dipole orientations of the single chlorophylls would be very useful. It is hoped that this information can be obtained by a combined effort of spectroscopic and molecular genetic approaches (11, 10, 18, 42), as proven effective in prokaryotic systems (43–45). In the case of minor chlorophyll proteins, besides the obvious function of light harvesting and energy transfer, a variety of regulatory mechanisms are associated (3, 46–48) whose structural frameworks need to be elucidated. As an example, a single point mutation of the Glu^{166} residue of CP29 abolished dicyclohexylcarbodiimide binding, thus allowing identification of a protonable residue potentially responsible for triggering of high energy quenching by low luminal pH (35). This approach needs to be extended to the other antenna proteins. In this work, we have reconstituted a CP24 complex from pigment extracts and the apoprotein overexpressed in *E. coli* as a step toward both the crystallization of the pigment-proteins and mutational analysis.

Native Versus Recombinant CP24: Is Recombinant CP24 "Better" Than the Complex Extracted from Leaves?—Our procedure yields recombinant CP24 proteins that exhibit many features of the native protein extracted from thylakoids: apparent molecular mass in green gels and density gradients, specificity of xanthophyll binding, characteristics of absorption, and circular dichroism spectra. Reconstitution of pigment-proteins from recombinant apoprotein and isolated pigments was previously performed in the case of the major LHCII protein (32, 42), of two LHCI subunits (49), and of the minor PSII subunit CP29 (33). In the latter case, it was possible to reproduce all of the biochemical and spectroscopic features of the native complex, thus allowing mutation analysis of chlorophyll-binding sites (12, 35). While the characteristics of CP29 are well known from previous studies (1, 10, 11), those of CP24 are ill defined. Since the first report (50), CP24 has been reported to have different chlorophyll *a*/*b* ratio, ranging from 0.9 to 1.6, depending on plant material and purification procedures (for a review, see Ref. 2), thus suggesting that this protein is unstable and loses pigments once extracted from thylakoids. This is possibly due to the lack of the short amphiphylic helix located near the C terminus of most Lhc proteins (40), as suggested from the results of C-terminal deletions in LHCII with impaired Chl binding (32, 51). Because we could not rely on the correspondence of the nCP24 preparation to the protein inserted in the thylakoid membrane, we refer to the case of CP29 as a model.

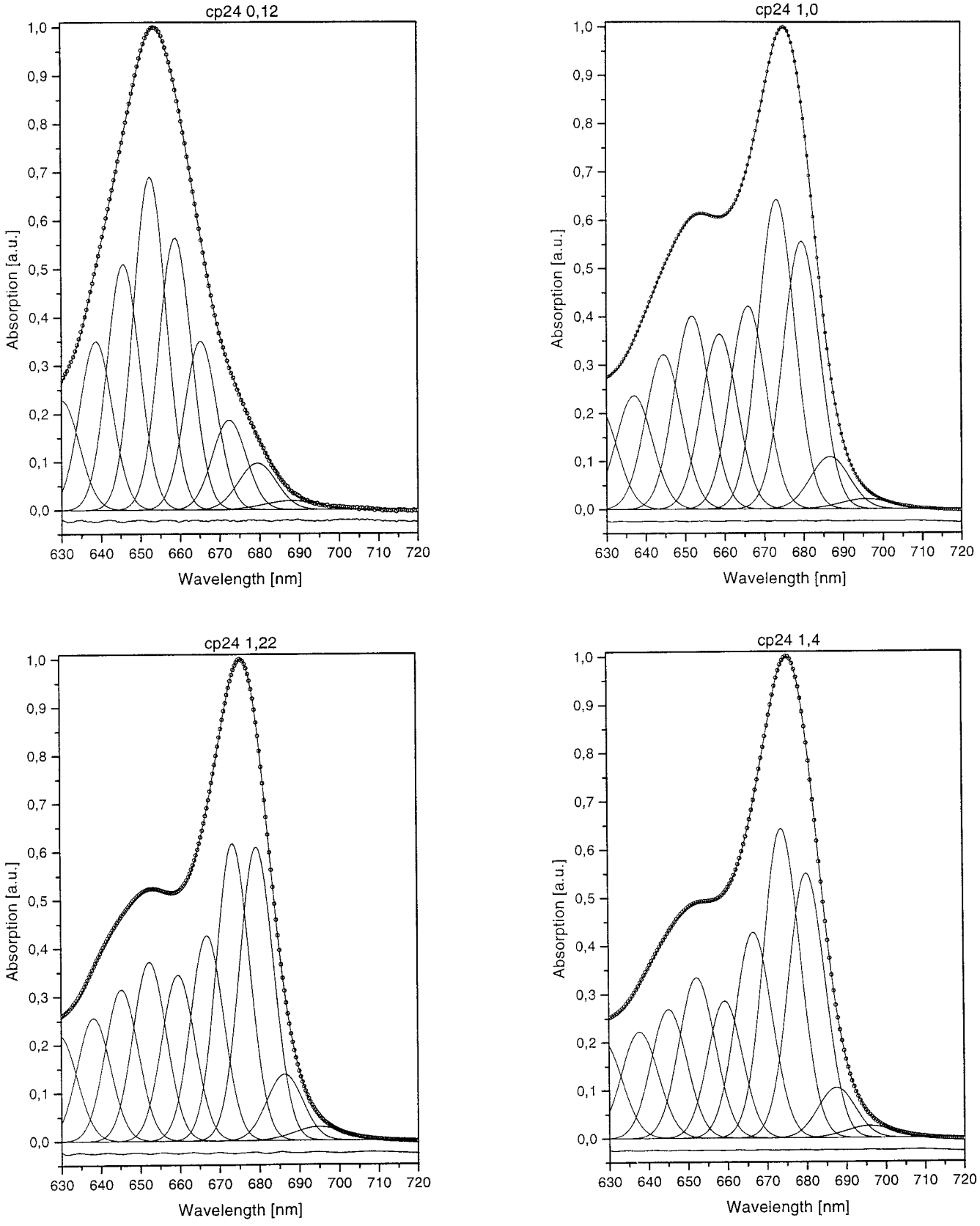

FIG. 9. **Gaussian subband analysis of absorption spectra of native and reconstituted CP24 measured at 300 K.** *Lower left*, nCP24 (native); *upper right*, rCP24 1.0; *lower right*, rCP24 1.4; *upper* left, rCP24 0.12. The residual plot is also shown *below* each plot for fitting accuracy (see also "Experimental Procedures").

As shown in Fig. 4, a family of rCP29 proteins was generated by refolding in the presence of pigments mixtures with different Chl *a*/*b* ratios (12, 33); increasing Chl *a*/*b* ratio yielded proteins with lower Chl *b* content, thus reaching the Chl *a*/*b* ratio of 3.0, corresponding to the ratio in the native protein, with an 8-fold excess of Chl *a* over Chl *b* during refolding. A rCP29

TABLE III

Gaussian decomposition parameters for native and reconstituted CP24 complexes with different Chl a/b ratios

See "Experimental Procedures" for method of analysis. The data are graphically represented in Fig. 9. The temperature was 300 K.

rCP24 Chl a/b = 0.12			rCP24 Chl a/b = 1.0			nCP24 Chl a/b = 1.22			rCP24 Chl a/b = 1.4		
λ_{max}	Area	FWHM	λ_{max}	Area	FWHM	λ_{max}	Area	FWHM	λ_{max}	Area	FWHM
nm	*%*	*nm*	*nm*	*%*	*nm*	*nm*	*%*	*nm*	*nm*	*%*	*nm*
630	4.2	10.1	628	2.3	10.8	629.5	3.3	10.3	628.5	2.7	11.3
639	11.9	9.6	637	7.2	10.8	638	7.8	9.9	638	7.4	11
646	17.5	9.6	644.5	10.2	10.6	645	9.7	9.6	645	9.0	10.6
652.5	23.6	9.6	652	12.7	10.6	652.5	11.6	9.9	652.5	11.3	10.6
659	19.4	9.6	658.5	11.4	10.6	659.5	10.7	9.9	659.5	9.3	10.1
665.5	11.9	9.6	666	13.3	10.6	667	13.1	9.6	666.5	14.7	10.8
672.5	6.6	9.9	673	20.5	10.6	673.5	18.9	9.6	674	22.1	10.8
679.5	3.8	11	679.5	17.9	10.8	679.5	19.1	9.9	680	18.8	10.6
			686.5	3.7	11.5	686	4.6	10.6	687.5	3.65	11
688	1.1	16	695.5	0.9	14.1	695	1.3	14.3	696	1.1	14.1

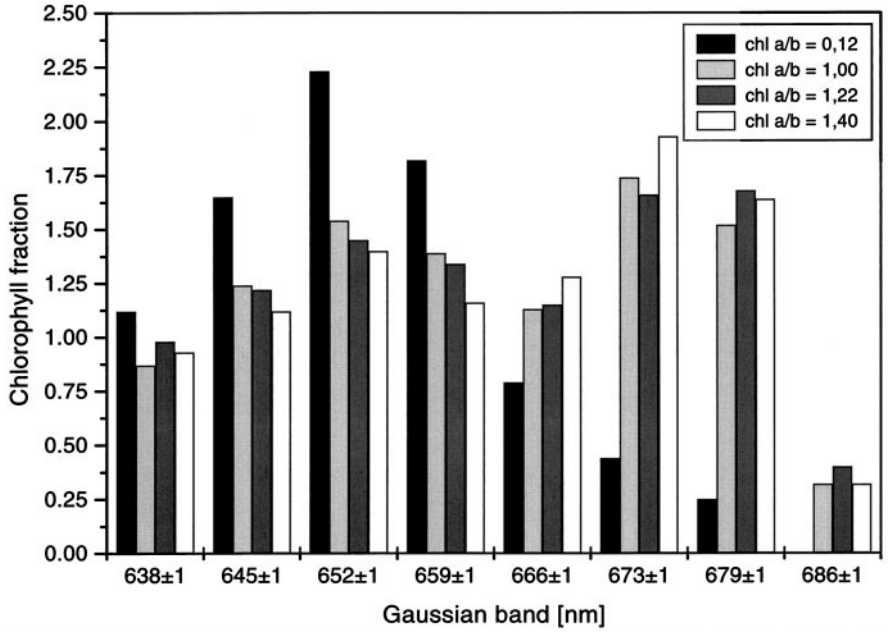

FIG. 10. **Histogram of areas for the different subbands as obtained from Gaussian deconvolution.** Spectra were normalized for the same total Chl a plus Chl *b* concentration as determined by HPLC analysis (see also "Experimental Procedures").

complex with increased Chl *a* content could only be obtained by using a Chl a/b ratio >20 during folding[2]; therefore, a plateau was obtained in the plot of Fig. 4, showing that between 8 and 20 a rCP29 with a Chl a/b ratio of 3 was obtained. This complex was carefully examined and found to be identical to the native complex by analysis with a number of spectroscopic and biochemical methods (12, 33, 35), thus suggesting that this protein's conformation is intrinsically more stable as compared with others with altered Chl *a versus* Chl *b* binding. Following this example, we could identify a similar behavior in the case of rCP24; Chl a/b in the complex rapidly increased from 0.12 to 1.0, and this value was maintained with an increasing Chl a/b ratio in the folding mixture between 2.0 and 5.8. rCP24 1.0 has a CD spectrum almost identical to nCP24, while other products show distinct differences in their CD such as blue or red shifts in the wavelength of the red-most negative signal. A similar behavior was previously recognized in the case of CP29, where products with altered Chl a/b ratio showed red or blue shifts in their red-most negative signal (12, 33). On the basis of the CD spectra and of the plot of Chl a/b ratio in the refolding mixture *versus* that in the complex, we propose that rCP24 1.0 corresponds to the undenatured CP24, while the nCP24 is partially denatured as a result of the purification procedures. This is not surprising, since purification of CP24 requires a preparative isoelectric focusing step in which complexes are subjected to low pH and high voltage during several hours, while the refolding procedure involves milder steps such as rapid FPLC separation and glycerol gradient ultracentrifugation. It appears that the more stable CP29 protein is not affected by purification procedures, since its spectroscopic properties remain the same irrespective of the procedure used (6, 52), while CP24 is quite sensitive to harsh purification steps as discussed above.

Chlorophyll Binding—Porphyrin binding of rCP24 1.0 has been determined; it binds 10 chlorophyll molecules per polypeptide and therefore five Chl *a* and five Chl *b*. This value of 10 is intermediate between those of LHCII (seven Chl *a* plus five Chl *b* (1, 34)) and of CP29 (six Chl *a* plus two Chl *b* (1)). Comparison of cDNA-deduced sequences shows that at least seven of eight residues reported to coordinate porphyrins in LHCII (34) are conserved, thus setting at seven the lower limit for Chl binding in CP24. The previously reported value of five chlorophylls per CP24 polypeptide (1) is therefore to be ascribed to pigment loss, since the same measurements yielded eight Chl in CP29 with both the native and recombinant protein (12, 33).

Carotenoid Binding and Specificity: Lutein Is Not Essential for Folding of CP24—Two xanthophyll molecules have been found in rCP24, irrespective to the Chl a/b ratio. This is in accordance with the case of CP29 (3, 12, 33) but different with respect to LHCII, where three rather than two xanthophyll molecules per polypeptide were found (53), suggesting that the presence of two xanthophyll binding sites might be a general

[2] E. Giuffra, D. Cugini, and R. Bassi, unpublished results.

feature of monomeric antenna complexes. Accordingly, monomerization of LHCII has been reported to be accompanied by carotenoid loss (54). A peculiar feature of CP24 with respect to the other PSII antenna proteins is the lack of neoxanthin (3). This feature was reproduced through *in vitro* reconstitution, further indicating that the procedure here described yields antenna proteins closely resembling their native state. LHCI proteins also lack neoxanthin when isolated from thylakoids (55), but Lhca1 and Lhca4 proteins, reconstituted by a different procedure, bound substantial amounts of neoxanthin (49). The functional reason for the absence of neoxanthin in CP24 and in LHCI proteins is presently unknown. CP24 can only be reconstituted in the presence of carotenoids consistently with previous reports with LHCII (18, 23, 32) and CP29 (33). However, there is only limited specificity in carotenoid binding. Any one of the carotenoids (neoxanthin, violaxanthin, or lutein) can be omitted from the reconstitution mixture in agreement with previous findings with LHCII from delipidated thylakoid proteins (23).

Xanthophyll Cycle Carotenoids in CP24—Recombinant CP24 does not bind neoxanthin; however, it forms a stable complex in the absence of lutein and therefore binds only violaxanthin. This result is particularly interesting, since it is different from what has been found with LHCII or CP29. The latter have an absolute requirement for lutein, although they can be interchanged with other xanthophylls (18, 23, 32, 33). Analysis of *Arabidopsis* mutants showed that increased amounts of other carotenoids can compensate for the absence of lutein (56).

It is tempting to correlate the capacity of violaxanthin to stabilize the pigmented complex of the CP24 apoprotein with the dominant role that CP24 is thought to play (together with CP29 and CP26) in the xanthophyll cycle (57, 58). These three complexes bind most of the violaxanthin in dark adapted plants (3) and of the zeaxanthin after exposure of leaves to excess light (57, 59). However, the violaxanthin to zeaxanthin conversion is more rapid and complete in CP24 and CP26 in comparison with CP29 (59, 60). Recent results with CP26 suggest that it shares the plasticity with respect to carotenoid binding described here for CP24 when the proteins are refolded *in vitro* (61). These data suggest that the ability of antenna proteins to participate in the xanthophyll-dependent light regulation mechanism correlates with the ability of the protein to accommodate violaxanthin within the carotenoid binding sites. The involvement of xanthophylls in the dissipation of excess light energy includes the deepoxidation of violaxanthin to yield zeaxanthin. This reaction is not yet fully understood (for a review see Ref. 2) particularly with respect to the location of the violaxanthin that forms the substrate to the deepoxidase, situated in the lumen.

Chlorophyll a and Chlorophyll b Contributions to the Absorption Spectrum—On the basis of the determination of the chlorophyll to protein stoichiometry, it is shown that each native monomer complex binds on an average five molecules of Chl *a* and five molecules of Chl *b*. When the *a*/*b* binding ratio is decreased, the amount of bound Chl *b* increases while that of Chl *a* decreases. It is important to note that the total average number of bound chlorophylls tends to remain constant at 10. In the extreme case here investigated, consisting of eight bound Chl *b* molecules and one bound Chl *a* molecule per polypeptide, a slight decrease of Chl binding of 0.9–1 molecules per polypeptide is detected, thus indicating that ectopic Chl *b* binding is only moderately defavored. This result suggests that CP24 has 10 Chl binding sites, several of which may be occupied by either Chl *a* or Chl *b*. This statement has some limitations, since at least one site remains largely empty if Chl *a* is not available, and another shows a very high affinity for Chl *a*, since it binds Chl *a* even where there is a 1000-fold excess of Chl *b*.

In order to reconstitute spectroscopically useful complexes, it is necessary to demonstrate that pigment binding occurs in a specific and "correct" way and that the complex is functionally competent. To this end, we performed two distinct kinds of experiment. First, the absorption/fluorescence relationship was analyzed by means of the Stepanov expression. This analysis demonstrated that thermal equilibration is essentially attained in both native and reconstituted complexes, which means that all pigments are coupled energetically even in the case of rCP24 0.12 and that the deviation from equilibration is rather small (possibly due to the a missing chlorophyll site). Secondly, by the fluorescence experiments, the excitation spectrum reveals that chlorophyll *b* is able to transfer excitation energy to Chl *a*. These observations, together with the similarity of rCP24 and the native complex in respect to many optical properties, strongly indicate that the pigment binding occurred "correctly" during the present reconstitution experiments.

In the remaining discussion, we will address the relevance of the present experiments to an understanding of the absorption characteristics of Chl *a* and Chl *b* in the antenna proteins of higher plants. In order to accurately describe room temperature absorption in the Qy region, taking into account reasonable values for the broadened bandwidths (9–11 nm at room temperature, 6–7 nm at 71 K) (62), eight Gaussian subbands are required. In the 630–665-nm region, where Chl *b* absorption is expected, four subbands, with wavelength positions at 639, 645, 653, and 659 nm were obtained. These values were previously reported in the case of the homologous proteins CP 29 and LHCII (12, 38), suggesting that the conclusions reached in the case of CP24 can be extended to the absorption of the whole Lhcb proteins. Independent evidence for the 648- and 655-nm transitions comes from photo-oxidation experiments in CP29 (12). In rCP24 0.12, all four subbands increase in intensity, while those at higher wavelengths are strongly decreased. It is clear that these former four subbands are associated with Chl *b* absorption. An interesting point that should be emphasized is that we detected only small changes in the wavelength positions of these four Chl *b* transitions by Gaussian analysis or second derivative analysis (not shown), although the average binding stoichiometry for Chl *a* changes from about 5 to 1 molecules per polypeptide. This was also true for the four Gaussian subbands in the 660–690-nm range (peaking at 666, 673, 679, and 686 nm) that are strongly decreased or absent in rCP24 0.12, and they can be associated to Chl *a* absorption. It should be noted that, although in rCP24 0.12 a single Chl *a* molecule is present per polypeptide, its absorption can be deconvoluted into three Gaussian subbands, *i.e.* 666, 673, and 679 nm. It follows that the number of spectral bands associated with Chl *a* in rCP24 0.12 is greater than the average number of bound Chl *a* molecules. This clearly indicates Chl *a* binding heterogeneity at the level of the different complexes. We therefore envisage each CP24 preparation to represent a mixed site population of bound Chl *a* molecules. A similar conclusion was obtained for Chl *b* in CP29 (12). This is in line with the conclusion discussed above that most Chl binding sites (all except for two) may bind either Chl *a* or Chl *b*.

The above conclusions, although mostly evident from the analysis of rCP24 0.12, which is partially affected in its pigment-pigment energy transfer due to an empty Chl site, are consistent with the characteristics of the fully functional rCP24 1.0, rCP24 1.4. The exact population binding ratio is expected to be determined, at least in part, by the Chl *a*/*b* ratio present during pigment-protein folding both *in vivo* and *in vitro*. This is in agreement with a previous work on intermittent light-grown plants in which lower Chl *b* availability caused a higher chlorophyll *a*/*b* ratio in CP29 and CP26 proteins (63). It is clear

that sites have different binding affinities for Chl *a versus* Chl *b*; absorption forms described by Gaussian subbands peaking at 666 and 673 nm are increased in their amplitude in rCP24 1.4 (six Chl *a versus* four Chl b) with respect to rCP24 1.0 (five Chl *a versus* five Chl *b*), implying that there are binding sites that have a low selectivity against Chl *b*. Another observation is that the amplitude of the 639-nm Chl *b* subband increases by 13% in rCP24 0.12 with respect to rCP24 1.0, while the increase in other Chl *b* forms ranges from 30 to 55%; this suggests that the 639-nm absorption is produced by a rather unique Chl *b* site and that the Chl *a* sites, where ectopic Chl *b* may be bound, do not provide the basis for such a strong blue shift as observed in Chl *b* absorption (Chl *b* spectrum in ether peaks at 645 nm). Charged groups interacting with π-orbitals might be responsible for this absorption (45).

In the end, protein-pigment interaction in different sites seems to play a dominant role in determining the absorption energies. That excitonic interactions, although weak, exist in CP24, as can be clearly observed by the behavior of the positive 428-nm (+) signal and of the 477 (−) signal in the CD spectra. This structure, attributed to Chl *a*-Chl *b* interaction (64), is present in native complex, and its amplitude is greatly increased in the complex binding one Chl *a* and eight Chl *b* molecules.

CONCLUSIONS

In this study, we have reconstituted the CP24 pigment-binding subunit of photosystem II from the overexpressed apoprotein and purified pigments. The recombinant protein binds five Chl *a*, five Chl *b*, and two xanthophyll molecules per mol of polypeptide and appears to be more stable with respect to the protein extracted from leaves, which loses pigments during purification. Recombinant CP24 has a different specificity in xanthophyll binding with respect to other members of Lhc family that can be important in the xanthophyll cycle photoprotection mechanism. This recombinant protein can be used for mutational analysis of both the polypeptide sequence and of the chromophore moiety. As an example of the latter approach, we show, by using recombinant proteins with altered chromophore composition, that the principle components for chlorophyll absorption can be described by four Gaussian subbands (639, 645, 653, and 659 nm) for chlorophyll *b* and four more (666, 673, 679, and 686 nm) for chlorophyll *a* in the CP24 spectrum.

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