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Use of Immobilized Light-Harvesting Chlorophyll a/b Protein to Study the Stoichiometry of Its Self-Association†

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ABSTRACT: D. J. Davis & E. L. Gross (1976) *Biochim. Biophys. Acta 449,* 554-564 previously observed that the light-harvesting chlorophyll a/b protein or chlorophyll protein complex II self-associated as determined by ultracentrifugation. We have determined the stoichiometry of complex formation by immobilizing the monomer on ethylenediamine-Sepharose 4B and determining the ability of immobilized protein to bind the free protein. The amount of soluble protein bound to the immobilized protein increased as the concentration of soluble protein increased. The binding was maximal between pH 7 and 8. The maximum binding was three molecules bound per one molecule of protein immobilized. These results indicate that a tetramer is the intrinsic structural unit of the light-harvesting chlorophyll a/b protein in the chloroplast membrane. Upon complex formation, the chlorophyll fluorescence was decreased without any spectral change. The maximum binding was approximately doubled upon addition of 0.5 mM $CaCl_2$ whereas 5 mM NaCl had no effect. Addition of $CaCl_2$ had no effect on the fluorescence of the monomer. The light-harvesting chlorophyll a/b protein can be isolated from a sodium lauryl sulfate extract of chloroplasts by affinity chromatography using the immobilized light-harvesting chlorophyll a/b protein.

The light-harvesting chlorophyll a/b protein (chlorophyll protein complex II or CP II[1] of Thornber (1975)) is one of three chlorophyll protein complexes of the chloroplast membrane. The other two are the core complexes of the two photosystems (Vernon et al., 1971). This protein has an important structural role as it contains 40-60% of the chlorophyll and protein of the chloroplast membrane (Thornber, 1975). It also contains all of the chlorophyll b of the chloroplast. It has also been implicated in the regulation by cations of the spillover of excitation energy from photosystem II to photosystem I (Davis & Gross, 1976; Prochaka & Gross, 1977). Membrane structural changes involving the stacking of the grana membranes are thought to be responsible for the changes in spillover (Murakami & Packer, 1971; Murata, 1971; Gross & Prasher, 1974; Anderson, 1975). We think that both the changes in spillover and membrane stacking are due to changes in the interaction of this protein with itself and with the other core complexes (see Arntzen & Ditto, 1976).

Davis & Gross (1975, 1976) showed both a concentration dependent and divalent cation dependent association of CP II. However, the stoichiometry of association and the binding constants were not determined. To answer this question, we have studied the association of soluble CP II with immobilized CP II.

Materials and Methods

Preparation and Immobilization of CP II. CP II was isolated from spinach by the methods of Kung & Thornber (1971) as modified by Davis & Gross (1975) (i.e., CP II was isolated from a 1% $NaDodSO_4$ extract of spinach chloroplasts by means of batchwise hydroxylapatite chromatography followed by ammonium sulfate precipitation). CP II containing from 50 to 100 μg of chlorophyll was dialyzed against distilled water and added to 1 mL packed volume of activated Sepharose 4B which had been coupled to 0.1 M ethylenediamine according to the method of Liberatore et al. (1976). A water-soluble

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[1] Abbreviations used are: CP II, chlorophyll protein complex II or light-harvesting chlorophyll a/b protein; $NaDodSO_4$, sodium dodecyl sulfate; Chl, chlorophyll.

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TABLE I: Summary of the Immobilization of CP II.[a]

CP II	Chlorophyll (μg)	Chlorophyll a/b
Added[b]	74.4	1.24
Filtrate[c]	1.0	
$NaDodSO_4$ extract[c]	2.6	
Immobilized[b]	71.4	1.18

[a] CP II (74.4 μg of chlorophyll) was mixed with 1 mL of ethylenediamine-Sepharose 4B. Immobilization was carried out in the dark at room temperature with 0.2 g of 1-ethyl-3-(dimethylaminopropyl)-carbodimide adjusted to pH 5.0 with dilute HCl. The filtrate and $NaDodSO_4$ extract contain all the CP II which was not covalently bound to the ethylenediamine-Sepharose. [b,c] Chlorophyll concentrations were determined by the method of Arnon (*b*) and by the absorbance at 436 nm (*c*).

carbodiimide was used to couple the CP II to the ethylenediamine-Sepharose 4B. Immobilization was conducted at room temperature at pH 5.0 for 2 h in the presence of 0.2 g of 1-ethyl-3-(dimethylaminopropyl)carbodiimide. The suspension was stirred to avoid protein–protein covalent linkages. CP II-Sepharose 4B was washed on a glass filter first with distilled water and then with 1% $NaDodSO_4$ to dissociate any CP II which was not covalently bound and finally with 50 mM Tris-Cl (pH 8.2). The gel was suspended in 30 mL of 50 mM Tris-Cl (pH 8.2) and stored at 5 °C. The control gel was processed by the same procedure as the CP II-Sepharose 4B but without the protein. The chlorophyll content of the immobilized CP II was determined according to the method of Arnon (1949).

Assay of Soluble CP II Binding with the Immobilized CP II. Binding experiments were conducted as follows unless otherwise described. Two milliliters of CP II (containing 2 to 8 μg/mL chlorophyll) was mixed in a small centrifugation tube with various amounts of the immobilized CP II. The final volume was 3 mL. The reaction mixtures were incubated in an ice bath for 2 h in the presence or absence of $CaCl_2$ with occasional shaking after which they were centrifuged at 5000*g* for 10 min at 5 °C. About 2 mL of the supernatant was transferred into a cuvette with a Pasteur pipet. The absorbance of CP II in the supernatant was measured at 436 nm with a Hitachi–Perkin-Elmer Model 139 spectrophotometer and the chlorophyll concentration was determined using the nonlinear absorbance vs. chlorophyll concentration curve of Davis & Gross (1976). Then, the supernatant and immobilized CP II were combined and adjusted to a final volume of 3 mL to which 0.3 mL of 10% $NaDodSO_4$ was added. The mixture was incubated at room temperature for 10 min with shaking and centrifuged at 5000*g* for 10 min at 5 °C. The absorbance of the supernatant was measured, corrected for the dilution by the detergent, and taken as the total CP II concentration. After the removal of the $NaDodSO_4$ by washing with 50 mM Tris-Cl (pH 8.2), the immobilized CP II was resuspended in the same buffer and the chlorophyll was extracted with 80% acetone.

Fluorescence Measurements of the Immobilized CP II. Five milliliters of the immobilized CP II suspension (containing 4.8 μg of chlorophyll) was filtered through a Millipore filter (25 mm diameter) with or without the addition of either 5 mL of CP II (containing 5.0 μg/mL chlorophyll) or 0.5 mM $CaCl_2$. The filter was cut to a width of 1.3 cm and exposed to the excitation beam at an angle of 45° in a fluorescence cuvette. Chlorophyll fluorescence spectra were measured using an Aminco-Bowman spectrofluorimeter at 20 °C. The excitation wavelength was 436 nm.

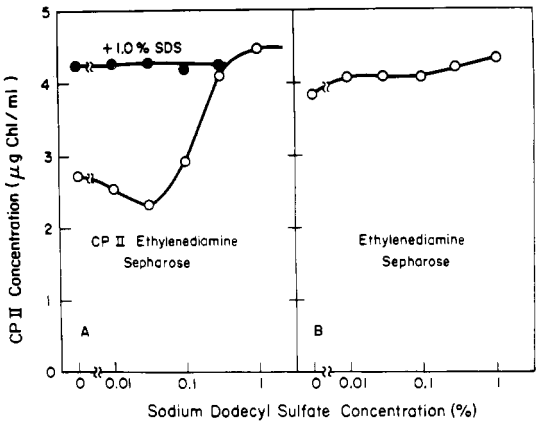

FIGURE 1: The effect of $NaDodSO_4$ on the binding of soluble CP II with the immobilized CP II. The immobilized CP II (5.58 μg of chlorophyll) (A) and the control gel (ethylenediamine-Sepharose 4B) (B) were incubated with CP II for 2 h at 10 °C. Chlorophyll concentrations of the supernatant were determined before (○) and after (●) the further addition of $NaDodSO_4$. A decrease in concentration corresponds to binding to the immobilized CP II.

Chemicals. Sepharose 4B and AH-Sepharose were obtained from Pharmacia. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl was obtained from the Storey Chemical Co. All other chemicals were of reagent grade. Double glass distilled water was used throughout these experiments.

Results and Discussion

Immobilization of CP II. Ethylenediamine was chosen as the spacer between the Sepharose 4B and the CP II for the following reasons. When molecules of longer chain length such as diaminohexane or diaminopropane were used, there was noncovalent hydrophobic binding of the CP II to the spacer. This is consistent with the observation of Thornber et al. (1967) that the surface of the protein must contain a considerable number of hydrophobic amino acids. On the other hand, when NH_3 was used for coupling, the amount of soluble CP II which could be bound to the immobilized CP II was decreased due to steric hindrance.

When the CP II was added to the ethylenediamine-Sepharose 4B in the presence of the carbodiimide, 96% of the CP II was bound without any change in the chlorophyll a/b ratio (Table I). $NaDodSO_4$ removed little chlorophyll protein from the gel. Therefore, the bound protein must have been monomeric. This result contrasts with the results of the sedimentation velocity experiments of Davis & Gross (1975) which showed that the protein was an oligomer at these concentrations. In contrast, in other studies in which the immobilization technique has been used to study binding (Chan, 1970; Chan & Mawer, 1972; Ikeda & Fukui, 1974) denaturing solvents removed those parts of the oligomeric proteins which were not covalently attached to the matrix. Our results suggest that there is a rapid dissociation of the protein which allows all of the subunits to be bound to the matrix in monomeric form.

Binding of the Soluble CP II with the Immobilized CP II. We determined the time course of the binding of soluble CP II with the immobilized CP II. Equilibrium was attained in 30 min under all conditions. Addition of Ca^{2+} ions increased the amount of CP II bound but Na^+ ions had no effect (data not shown). These results agree with those of Davis & Gross (1975, 1976).

Figure 1A shows the effect of the addition of $NaDodSO_4$ on the binding of soluble CP II with the immobilized CP II. A decrease in the amount of CP II in the supernatant repre-

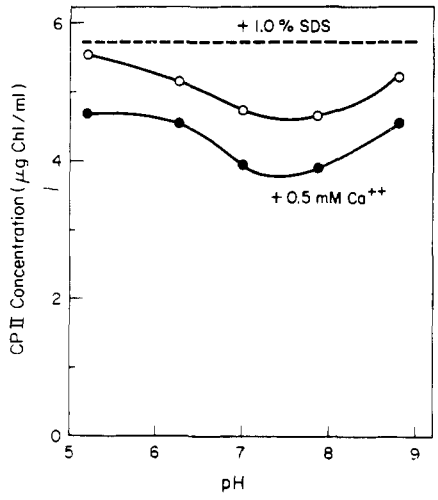

FIGURE 2: The effect of pH on the binding of soluble CP II to the immobilized CP II. The immobilized CP II (2.37 μg of chlorophyll) was incubated with the soluble CP II at the pH values indicated. The pH was adjusted with 0.12 M Tris-succinate buffer. The dashed line was obtained by extrapolating the data from the pH 7–9 region since chlorophyll bleaches in acidic solutions of $NaDodSO_4$.

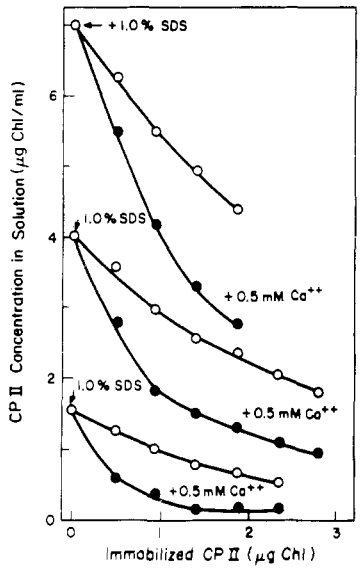

FIGURE 3: Binding of soluble CP II with immobilized CP II. Various amounts of the immobilized CP II were incubated with the soluble CP II in the absence (○) and presence (●) of 0.5 mM $CaCl_2$. The total CP II concentration is depicted by the points where the immobilized CP II = 0 and were obtained from the average absorbance at 436 nm with 1.0% $NaDodSO_4$ as described in the Materials and Methods section. The variance was ±2%. All samples contained 10 mM Tris-Cl (pH 8.2).

sents the amound bound. In the absence of $NaDodSO_4$, one molecule of immobilized CP II bound 0.83 molecule of soluble CP II when the soluble CP II concentration was 2.7 μg/mL chlorophyll. Increasing the $NaDodSO_4$ concentration to 0.03% caused a slight increase in CP II binding. However, the binding was reduced to zero above 0.2% $NaDodSO_4$. Addition of 0.3 mL of 10% $NaDodSO_4$ released all of the bound CP II and all of the original absorbance was recovered in the supernatant.

We also tested the binding of the soluble CP II to ethylenediamine-Sepharose 4B which has not been complexed with CP II (Figure 1B). No binding was observed. This rules out a nonspecific interaction of the CP II with the gel.

The pH dependence of the binding of soluble CP II with immobilized CP II was determined (Figure 2). Again, the binding is shown by the decrease in absorption of 436 nm. The

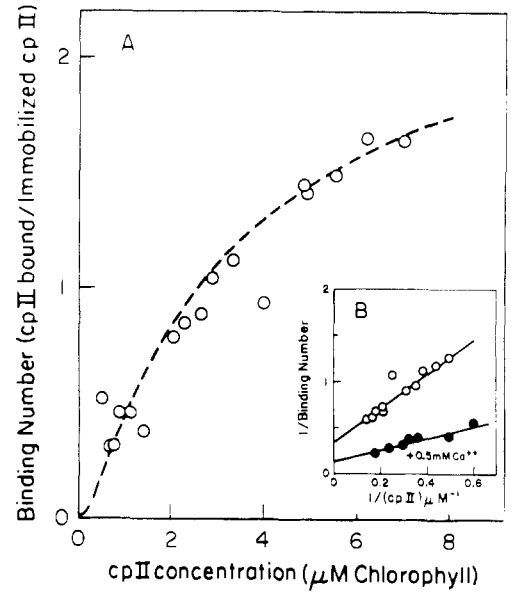

FIGURE 4: The dependence of binding on CP II concentration. The data of Figure 3 for CP II binding were plotted linearly (A) and double reciprocally (B) against the equilibrium CP II concentration in the absence (○) and presence (●) of 0.5 mM $CaCl_2$. The binding number can be calculated by dividing the concentration of bound CP II by the amount of immobilized CP II. The chlorophyll concentration was calculated using a molecular weight of 900. The broken line shows the best fit of the data derived from a model of monomer–tetramer association. The binding constant was 8×10^{18} M^{-3}. The analytical procedures and definitions are described in the text.

binding is maximal between pH 7 and 8 and falls off at higher and lower pH values. The same pH profile is observed in the presence of 0.5 mM $CaCl_2$. These results agree very closely with the pH profile observed for the Na^+-induced changes in chloroplast fluorescence and light scattering, both of which have proven to be parameters indicative of membrane stacking (Gross & Prasher, 1974). Therefore, these data also give further evidence for the idea that chloroplast membrane stacking may occur through the intramembrane association of CP II units.

Results of binding experiments at three different CP II concentrations are shown in Figure 3. Initial slopes were calculated to be 0.92, 1.05, and 0.65 mol of soluble CP II bound per mol of immobilized CP II at initial concentrations of soluble CP II of 1.5, 4.0, and 7.0 μg/mL chlorophyll, respectively. This concentration-dependent shift in the binding would suggest that the law of mass action is dominant in the reversible CP II association. Figure 3 also shows an increased binding of the soluble CP II in the presence of Ca^{2+} ions suggesting the formation of larger polymeric units. This is consistent with the findings of Davis & Gross (1975, 1976).

We used these data to determine the relationship between the binding number and the CP II concentration in solution (Figure 4). The binding number was calculated from the decrease in absorption at 436 nm. The free CP II concentration represents the equilibrium concentration since we have described that the system reached equilibrium after 30 min of incubation.

In an analysis of an associating system in which the components are in rapid equilibrium, one of the major problems is that it is difficult to determine a definite stoichiometry (Frieden, 1971). One of the advantages of using the immobilization technique is that a maximum binding number can be determined from a double reciprocal plot (Figure 4B) of the data shown in Figure 4A. The aggregation state of the polymer

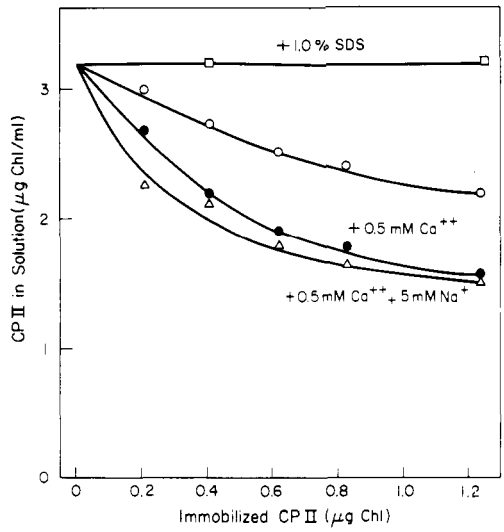

FIGURE 5: The effect of cations on CP II binding. Various amounts of the immobilized CP II were incubated with soluble CP II in the absence (○) and presence of 0.5 mM $CaCl_2$ (●) or 0.5 mM $CaCl_2$ + 5 mM NaCl (△). The total concentration of CP II (□) was determined by the addition of $NaDodSO_4$ as described in the Materials and Methods section.

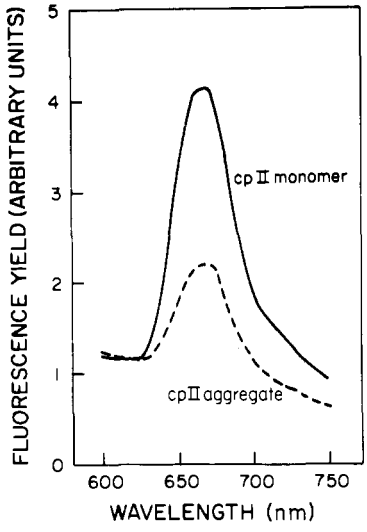

FIGURE 6: Fluorescence spectra of the immobilized CP II. Fluorescence spectra for the immobilized CP II were measured before (—) and after (···) the binding with soluble CP II. Conditions were as described in the Materials and Methods section.

is equal to the binding number + 1. This takes into account the immobilized subunit. Thus, the double reciprocal plot (Figure 4B) shows that CP II forms a tetramer in the absence of $CaCl_2$.

The abscissa of Figure 4 shows the total concentration of CP II in solution. This may consist of a mixture of monomers, dimers, tetramers, and other species. In order to determine the mechanism of association and the binding constants, we analyzed our data according to the method of Valdes & Ackers (1977). The CP II monomer $(CP\ II)_1$ is assumed to be in equilibrium with the polymer, n-mer, $(CP\ II)_n$. In addition, there may be intermediates, j-mers $(CP\ II)_j$. The total concentration of CP II in solution, $(CP\ II)_{total}$, and the immobilized CP II $(CP\ II)_{im}$ can be represented by the following formulas (eq 1-3)

$$(CP\ II)_{total} = \sum_{j=1}^{n} j(CP\ II)_j = \sum_{j=1}^{n} jk_j(CP\ II)^j \qquad (1)$$

$$(CP\ II_{im})_{total} = (CP\ II_{im}) \sum_{j=1}^{n} k_j(CP\ II)^{j-1} \qquad (2)$$

$$\text{binding no.} = \frac{\left[\sum_{j=1}^{n} (j-1)K_j(CP\ II)^{j-1} - (CP\ II)_{im}\right]}{(CP\ II_{im})_{total}} \qquad (3)$$

where k_j is used to designate the association constant between $(CP\ II)_1$ and $(CP\ II)_j$.

Computer models were made using one of two assumptions: (a) the association proceeds directly from monomer to tetramer and (b) the association proceeds from monomer to dimer to tetramer. The best fit was obtained using the first model. The calculated association constant was $0.8 \times 10^{19}\ M^{-3}$.

In this analysis, we have made the assumption that the association constants for the immobilized molecules are the same as for the molecules in solution. This has proven to be untrue for hemoglobin (Antonini et al., 1974) in which the association constants for the immobilized molecules were lower than for the molecules in solution. It is possible that our data could be affected in the same way.

In conclusion, the results of Davis & Gross (1975, 1976), which show a concentration-dependent increase in the sedimentation coefficient of CP II, can be explained on the basis of a reversible self-association with a rapid equilibrium and with the association constant calculated above (Cox, 1969).

In the presence of Ca^{2+} ions, the binding number was calculated to be 7.7 mol of CP II bound per mol of CP II immobilized (Figure 4B). The most reasonable interpretation is that an octamer is formed. However, the results obtained in the presence of Ca^{2+} ions disagree with the earlier results of Davis & Gross (1975, 1976) in two respects. First, the total binding number did not decrease at higher CP II concentrations (Figure 4). In contrast, Davis & Gross observed that Ca^{2+} ions increased the sedimentation coefficient to 12.3 S at low CP II concentrations but only to 10 S at CP II concentrations greater than 5 μg/mL chlorophyll. We reconcile these discrepancies using the following model. At low CP II concentrations, the only form observed in the absence of Ca^{2+} ions is the monomer. Addition of Ca^{2+} ions allows maximal octamer formation. At higher CP II concentrations, both monomer and tetramer exist in the absence of Ca^{2+} ions. We postulate that upon addition of Ca^{2+} ions, only the monomer can associate to form the octamer (i.e., the tetramer is not a subunit of the octamer). In fact, there is competition between the tetramer and the octamer resulting in an intermediate value for the sedimentation coefficient. Theoretical models of this type have been discussed by Cox (1969) and Cann (1970).

Also, we did not observe any effect upon addition of NaCl (in the presence of $CaCl_2$; see Figure 5). In contrast, Davis & Gross (1976) observed an increase in the sedimentation coefficient to a value of 12.3 S under these conditions. Possibly Na^+ ions affect the competition between the tetramer and the octamer.

Fluorescence of the Immobilized CP II. One of the principal advantages of immobilization is that the properties of the monomer may be studied under conditions where reassociation is prevented (Chain, 1970; Chan & Mawer, 1972; Ikeda & Fukui, 1974). Studies with the immobilized CP II show that the fluorescence yield of the monomer is very high (Figure 6). Upon incubation with soluble CP II, the fluorescence yield decreases to 43% of that of the monomer without any change in the emission spectrum. The emission spectrum is the same as that obtained by Davis & Gross (1976) for CP II in solution. Actually, the true fluorescence decrease which occurs on polymer formation may be greater than the observed value

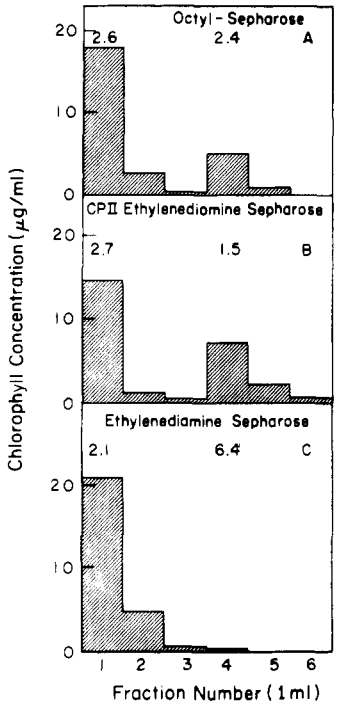

FIGURE 7: Affinity chromatography using the immobilized CP II. The chloroplast membrane was solubilized with a 1% solution of $NaDodSO_4$ and centrifuged. The supernatant was diluted to 0.03% $NaDodSO_4$ with 10 mM Tris-Cl (pH 8.2) and applied to one of the following columns: (A) octyl-Sepharose, (B) immobilized CP II, and (C) ethylenediamine-Sepharose 4B. The columns had been equilibrated with 10 mM Tris-Cl (pH 8.2). The $NaDodSO_4$ concentration was raised to 1.0% prior to the fourth fraction. The numerals in the figure show the chlorophyll a/b ratios for fractions 1 and 4. The material eluted in the fourth fraction represents the material bound to the column.

because the CP II content of the cuvette has been increased by the amount of soluble CP II bound. One possible explanation is that resonance energy transfer is increased in the aggregate (Förster, 1960). This may allow additional opportunities for deexcitation. Addition of Ca^{2+} ions had no effect on the fluorescence of the CP II monomer (data not shown). This suggests that Ca^{2+} ions do not produce large structural changes in the CP II monomer. In support of this, we observed no changes in the secondary structure of CP II as measured using far-ultraviolet circular dichroism (M. Takahashi & E. L. Gross, unpublished data). Therefore, the Ca^{2+}-induced decreases in chlorophyll fluorescence observed by Davis & Gross (1976) and Arntzen & Ditto (1976) must be a result of aggregation.

Affinity Chromatography Using the Immobilized CP II. We wanted to determine whether we could use immobilized CP II to isolate more CP II from a $NaDodSO_4$ extract of the chloroplast membranes. The results are presented in Figure 7B. The first three fractions contain material which was not bound to the column. Fraction 4 contains material which was bound to the column initially and which was removed with 1% $NaDodSO_4$. When CP II–ethylenediamine Sepharose 4B was used (Figure 7B), fraction 4 contained material with a low chlorophyll a/b ratio. Since the chlorophyll a/b ratio was 1.5 rather than the value of 1.1 observed with pure CP II, we estimate there may be up to 8% contamination with other pigment proteins. The core complexes for the two photosystems are the most likely candidates since it has been shown that CP II can bind to them (Arntzen et al., 1976; E. L. Gross, unpublished observations). The CP II obtained by this method can be purified by putting it through the column two more times. The observation that very little of the other pigment protein complexes are bound to immobilized CP II indicates that it does not bind protein in a nonspecific manner.

On the other hand, when ethylenediamine-Sepharose 4B was used (Figure 7C), no binding was observed. These data agree with those presented in Figure 2. Octyl-Sepharose (Figure 7A) bound some of the detergent extract but no fractionation was observed.

Conclusions

We have shown that the tetramer is the predominant polymeric form of CP II in the absence of $CaCl_2$. The association pattern is monomer to tetramer and the association constant is 0.8×10^{19} M^{-3}. The predominant form is an octamer in the presence of $CaCl_2$.

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